NEW INSIGHTS INTO REDUCTIVE DETOXIFICATION OF CHLORINATED SOLVENTS AND RADIONUCLIDES

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Kelly Elizabeth Fletcher

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Approved by:

Dr. Frank E. Löffler, Advisor
School of Civil and Environmental Engineering
Georgia Institute of Technology

Dr. Jim C. Spain
School of Civil and Environmetnal Engineering
Georgia Institute of Technology

Dr. Kurt D. Pennell
School of Civil and Environmental Engineering
Tufts University

Dr. Martial Taillefert
School of Earth and Atmospheric Sciences
Georgia Institute of Technology

Dr. Joseph B. Hughes
School of Civil and Environmental Engineering
Georgia Institute of Technology

Date Approved: September 15, 2010

To my husband John Hoyt

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

1,1,2,2-TeCA	1,1,2,2-tetrachloroethane
1,1,2-TCA	1,1,2-trichloroethane
1,1,1-TCA	1,1,1-trichloroethane
1,1-DCA	1,1-dichloroethane
1,2-D	1,2-dichloropropane
1,2-DCA	1,2-dichloroethane
AKIE	apparent kinetic isotope effect
APS	Advanced Photon Source
BDI	Bio-Dechlor INOCULUM
BLAST	basic local alignment tool
bp	base pair
cDNA	complimentary DNA
CERCLIS	Comprehensive Environmental Response, Compensation, and Liability Information System
cis-DCE	*cis*-1,2-dichloroethene
CSIA	compound specific stable carbon isotope analysis
DCE	dichloroethene
DNA	deoxyribonucleic acid
DNAPL	dense nonaqueous phase liquid
DOE	U.S. Department of Energy
e^-	electron
EGDY	East Gate Disposal Yard
Eh	redox potential
EPA	U.S. Environmental Protection Agency
ERH	electrical resistance heating
EXAFS	extended X-ray absorption fine structure
FID	flame ionization detector
FT	Fourier transformed
GC	gas chromatograph
HPLC	high performance liquid chromatography
IFC	Integrated Field-Scale Subsurface Research Challenge
LB	Luria Bertani
K_h	Dimensionless Henry's constant
kJ	kilojoule
MCL	maximum contaminant level
mol%	mole percent
MW	molecular weight
PCE	tetrachloroethene
PCR	polymerase chain reaction
PDB	Vienna Pee Dee Belemnite
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
ppb	part per billion

PVC	polyvinyl chloride
qPCR	quantitative real-time PCR
RDase	reductive dehalogenase
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
sp.	species (singular)
spp.	species (plural)
TCE	trichloroethene
trans-DCE	*trans*-1,2-dichloroethene
VC	vinyl chloride
vol/vol	volume per volume
wt/vol	weight per volume
wt/wt	weight per weight
XANES	X-ray absorption near edge structure

B	biodegradation extent
C_0	initial number of moles of the parent compound per bottle
$C_{0,correct}$	corrected number of moles of the parent compound per bottle that were available for dechlorination at time t
$C_{r,t-1}$	cumulative number of moles of the parent compound that were removed per bottle due to sample collection at time t-1
C_t	number of moles per bottle of the parent compound at time t
$C_{t,correct}$	corrected number of moles of the parent compound present per bottle at time t
DE	dechlorination extent
f	molar fraction remaining at time t
$M_{consumed}$	number of moles of electron equivalents consumed for reductive dechlorination
M_{DCEs}	sum of the moles of DCEs
M_{ethene}	number of moles of ethene
M_{TCE}	number of moles of TCE
M_{tot}	the total number of moles of chlorinated ethenes and ethene
M_{VC}	number of moles of VC
n	number of carbon atoms in the molecule
t	time
x	number of carbon atoms in the reactive position
z	the number of indistinguishable reactive sites

$\Delta\delta^{13}C$	change in the carbon isotope composition from time 0 to time t
$\delta^{13}C_0$	carbon isotope composition at time 0
ε_{bulk}	bulk carbon isotope enrichment factor
$\varepsilon_{reactive}$	reactive position specific carbon isotope enrichment factor

SUMMARY

Chlorinated compounds and radionuclides are toxins and widespread environmental contaminants. Under anoxic conditions, naturally occurring bacteria are capable of the detoxification of chlorinated compounds and the immobilization of the radionuclide uranium. Chlorinated compounds are detoxified via reductive dechlorination to non-toxic products and uranium is immobilized via the reduction of soluble U(VI) to insoluble U(IV). Bioremediation exploits these metabolic processes for the clean-up or containment of contaminants and has been successfully employed in situ. To optimize bioremediation strategies, additional information regarding the biology of the microbes catalyzing these reactions is needed.

Physicochemical remediation techniques such as thermal treatment have been shown to remove chlorinated solvent mass from source zones. Thermal treatment has also been suggested to increase electron donor availability for dechlorinators and therefore, interest has developed in coupling bioremediation with thermal treatment. In this study, the potential risks and benefits of coupling thermal treatment with bioremediation for the remediation of chlorinated ethene contaminated sites was investigated. Experiments confirmed that thermal treatment increases electron donor availability, but results also demonstrated that the majority of reducing equivalents were consumed in the competing process of methanogenesis rather than reductive dechlorination. Based on these results, during the field-scale application of thermal treatment, biostimulation should be applied even when electron donor is released during thermal treatment.

In order to demonstrate that bioremediation is occurring in situ, multiple lines-of-evidence should be provided. Two approaches for monitoring chlorinated solvent bioremediation were investigated, molecular, PCR-based techniques and compound-specific isotope analysis (CSIA). Whereas *Dehalococcoides* (*Dhc*) gene copy abundances correlated with dechlorination activity, gene expression was up-regulated under conditions inhibitory to dechlorination. Results from CSIA established that the isotope effects associated with dechlorination reactions catalyzed by unique *Dhc* populations were similar. These results advanced understanding of *Dhc* biology and have practical implications for bioremediation monitoring. The up-regulation of *Dhc* reductive dehalogenase (RDase) gene expression in the absence of measurable dechlorination activity suggests that this up-regulation is a general *Dhc* stress response. Because the detection of *Dhc* gene transcripts does not necessarily indicate that active dechlorination is occurring, monitoring of *Dhc* gene transcripts may not provide reliable information regarding biodegradation activity. Based on the consistent isotope effects determined with CSIA, CSIA may be a powerful tool for quantitatively assessing biodegradation extent in situ.

Previous studies have reported that gram positive *Desulfitobacterium* are present at uranium contaminated sites and U(VI) reduction by multiple *Desulfitobacterium* isolates was investigated. The results of these experiments demonstrated that U(VI) reduction is a shared feature of members of the *Desulfitobacterium* genus. Furthermore, whereas nearly all U(VI)-reducing bacteria have been reported to produce uraninite (UO_2), the product of U(VI) reduction by *Desulfitobacterium* isolates was a unique form of insoluble mononuclear U(IV). Although both abiotic and biotic factors can influence

the form of the reduced product, the production of mononuclear U(IV) rather than UO_2 suggests that *Desulfitobacterium* may employ a unique biomolecular mechanism for U(VI) reduction.

CHAPTER 1

INTRODUCTION

1.1 Background

Chlorinated compounds such as chlorinated ethanes, ethenes, and propanes are widespread environmental contaminants (1). Bioremediation of chlorinated compounds under anoxic conditions relies on reductive dechlorination, the removal of a chlorine atom with the simultaneous addition of electrons to a molecule (2). Specifically, chlorinated ethenes undergo sequential reductive dechlorination where tetrachloroethene (PCE) is transformed via the intermediates trichloroethene (TCE); *cis*-1,2- or *trans*-1,2-dichloroethene (*cis*-, or *trans*-DCE); and vinyl chloride (VC) to yield non-toxic ethene and inorganic chloride (1, 3). Chlorinated ethanes and propanes possess chlorine substituents on adjacent, saturated carbon atoms and can undergo dichloroelimination wherein, e.g., 1,2-dichloroethane (1,2-DCA) is converted to ethene and 1,2-dichloropropane (1,2-D) is converted to propene. Numerous organisms dechlorinate PCE and TCE to *cis*-DCE, but *Dehalococcoides* (*Dhc*) spp. are the only known microorganisms capable of transforming DCE isomers and VC to ethene, although not all *Dhc* isolates are capable of metabolic VC dechlorination (4). *Dhc* spp. are also capable of dichloroelimination of chlorinated ethanes and propanes (4-5). For successful bioremediation with *Dhc* to occur in situ, viable *Dhc* capable of complete dechlorination must be present and hydrogen, the only known electron donor for *Dhc*, must be available. Furthermore, subsurface conditions must be favorable for *Dhc* activity (e.g., anaerobic

conditions, circumneutral pH). If favorable conditions exist for *Dhc* activity but *Dhc* are not present or hydrogen is limiting dechlorination activity, the subsurface can be bioaugmented (i.e., amended with *Dhc*) or biostimulated (i.e, amended with electron donor) (6).

Bioremediation using *Dhc* has been shown to effectively contain chlorinated solvent plumes (1, 6-7), but due to low aqueous solubility and high molecular weight, some chlorinated compounds, particularly PCE and TCE, may be present in the subsurface as dense nonaqueous phase liquids (DNAPLs) (8). Physicochemical remediation techniques, such as surfactant or cosolvent flushing, chemical oxidation, or thermal treatment remove DNAPL mass significantly faster than bioremediation alone (9-10); however, these techniques to do not typically remove 100% of contaminant mass (9, 11) and therefore, interest has developed in coupling bioremediation with aggressive physicochemical source zone remediation techniques (9-10, 12).

Coupling thermal treatment with bioremediation has been suggested as a promising approach for the remediation of chlorinated ethene contaminated sites (13). During thermal treatment, subsurface temperatures are raised while soil vapors are extracted to remove chlorinated solvents. Interestingly, thermal treatment also releases organic carbon from subsurface materials (13), potentially increasing electron donor availability for dechlorinators (13-14). In order for dechlorinating populations rather than their competitors (e.g., methanogens) to consume available electron donor, dechlorinators must both be present and actively respiring chlorinated solvents in situ. *Dhc* capable of complete dechlorination to ethene are unlikely to be actively dechlorinating at temperatures greater than 30°C (4, 15), but during thermal treatment, the target zone is

typically heated to temperatures of 100°C or greater (16). Even so, subsurface temperatures decrease with distance away from the heated treatment zone, and therefore, dechlorination may occur in the perimeter of the heated zone even during treatment. Furthermore, spore-forming, PCE-to-*cis*-DCE dechlorinating organisms such as *Clostridium bifermentans* strain DPH-1 (17) may recover activity following subsurface cooling. The potential for the recovery of *cis*-DCE and VC dechlorination by *Dhc* following exposure to elevated temperatures is unknown.

In order to demonstrate that in situ reductive dechlorination is occurring, multiple lines of evidence are required, usually including contaminant concentration measurements and results from molecular analyses (6-7). Molecular assays targeting *Dhc* biomarkers, the *Dhc* 16S rRNA gene and the *bvcA*, *tceA*, and *vcrA* reductive dehalogenase (RDase) genes, are specific and sensitive (18-19). Furthermore, temporal changes in gene copy numbers have been shown to correlate with dechlorination activity in many instances (6-7, 20-23). Despite these measured correlations, quantification of gene copies does not differentiate viable, dechlorinating *Dhc* cells from inactive or nonviable cells (18, 24). Quantification of gene transcripts is generally considered to provide a more direct measurement of cellular activity (1, 18) and a number of studies have demonstrated that *Dhc* RDase transcription correlates with metabolic activity during active dechlorination (18, 20, 24-29). One recent study, however, demonstrated that transcription is not predictive of dechlorination activity under inhibitory oxygen concentrations (18). Therefore, studies are required to determine if results from molecular analyses accurately correlate with dechlorination activity under the inhibitory conditions likely present during thermal treatment.

Compound specific stable carbon isotope analysis (CSIA) has been used to demonstrate that in situ dechlorination activity is occurring (30-34), but CSIA can also be used to estimate dechlorination extent (35). In order to predict dechlorination extent, accurate bulk carbon isotope enrichment factors (ε_{bulk}) must be known (35). Carbon ε_{bulk} factors have been determined for the reductive dechlorination of select chlorinated compounds by some pure and mixed cultures (36-37). However, ε_{bulk} factors for some reactions have never been determined (e.g., dichloroelimination of 1,2-D) and the determined ε_{bulk} factors vary widely for other reactions (e.g., reductive dechlorination of TCE) (36). The variability of ε_{bulk} factors for reactions catalyzed via the same mechanisms may be due to organism- or RDase-specific differences, but, to date, no studies have attempted to determine whether individual RDases generate distinct isotope effects.

Subsurface uranium contamination exists mainly due to the inappropriate disposal of uranium during historical uranium processing for the production of weapons (1, 38). In general, oxidized uranium, U(VI), is soluble and mobile whereas reduced uranium, U(IV), forms the insoluble uraninite precipitate (UO_2); therefore, bioreduction of U(VI) is a promising approach for the prevention of uranium plume migration (39). Research on the bioreduction of U(VI) has focused on gram negative organism even though a wide variety of microbes are capable of the reduction of U(VI) and studies have demonstrated that gram positive bacteria are also present at U(VI) contaminated sites (38, 40-42). *Desulfitobacterium* spp. have been identified at multiple U(VI) contaminated sites (41-43), but only one *Desulfitobacterium* isolate has been implicated in U(VI) reduction (44). Furthermore, the product of U(VI) reduction by *Desulfitobacterium* has never been

identified, even though recent studies have shown that U(IV) products other than UO_2 (e.g., U(IV)-citrate, ningyoite) may be generated from U(VI) reduction (45-47).

1.2 Thesis Rationale

The use of bioremediation for the treatment of chlorinated solvent- and uranium-contaminated sites has become increasingly accepted. Even so, key knowledge gaps exist with regard to i) the potential risks and benefits of coupling bioremediation with thermal treatment, ii) the accuracy of molecular tools and stable carbon isotope analysis for the assessment of bioremediation success, and iii) the range of organisms catalyzing U(VI) reduction and the identity and characteristics of the reduced products. The specific objectives of this research and the rationales for these objectives are described below. The results of the objectives are detailed in Chapters 3-8 and the overall conclusions from this work are presented in Chapter 9.

1.2.1 Objective 1: Evaluate the Potential for Coupling Thermal Treatment with Bioremediation for the Remediation of Chlorinated Ethene-Contaminated Sites

Thermal treatment releases organic carbon from the subsurface matrix, potentially increasing electron donor availability for dechlorinating organisms (13-14). The released carbon has been hypothesized by Friis et al. (2006) to be in the form of long chain fatty acids, which *Dhc* are not known to use as either a carbon source or as an electron donor. Therefore, further studies are required to determine whether the release of organic carbon caused by thermal treatment fuels reductive dechlorination or, perhaps, competing

metabolic pathways, such as methanogenesis. Chapter 3 describes insights into the availability of electron donor for dechlorinators following thermal treatment.

During thermal treatment, the subsurface is heated to temperatures approaching 100°C or higher, but complete dechlorination to ethene has only been reported to occur at temperatures from 10-30°C (12, 15, 48). Therefore, based on current reports, it is unlikely that dechlorination will occur in the treatment zone during thermal remediation; however, dechlorination may occur at the moderately elevated temperatures in the perimeter zone, or, if dechlorinators can recover activity following exposure to elevated temperatures, dechlorination may also occur during or after cool-down. PCE-dechlorinating spore-forming organisms, such as *Clostridium bifermentans* strain DPH-1 (17) may play a relevant role for initiating dechlorination following cooling from elevated temperatures, but the potential for the recovery of *cis*-DCE and VC dechlorination by *Dhc* is unknown. Chapter 4 describes results of a study investigating dechlorination and spore-formation in strain DPH-1. Chapter 5 describe the results of experiments conducted to determine both the maximum temperature at which complete dechlorination to ethene may occur as well as the potential for the recovery of dechlorination activity following exposure to inhibitory temperatures. During the experiments conducted to assess the maximum temperature for dechlorination, *Dhc* populations were also monitored using molecular techniques to determine how *Dhc* gene and transcript copy abundances are affected by inhibitory conditions.

1.2.2 Objective 2: Evaluate Available Methods for Monitoring *Dhc*

Dhc gene and transcript copy abundances have generally been shown to be predictive of dechlorination activity during active dechlorination (6-7, 20-23); however,

only one study has examined gene and transcript copy abundances under conditions inhibitory for dechlorination (18). In fact, as demonstrated in the previous study, gene transcript copy abundances were not predictive of dechlorination activity under inhibitory conditions (18). In Chapter 5, the effect of inhibitory temperatures on gene copy and transcript abundances was examined to determine whether results from molecular assays correlate with dechlorination activity under the inhibitory conditions present during thermal treatment.

CSIA has previously been used to demonstrate in situ dechlorination activity and ε_{bulk} factors for dechlorination of chlorinated solvents have been derived for multiple mixed and pure cultures (30-34); however, no ε_{bulk} factors have been determined for the dichloroelimination of 1,2-D nor are ε_{bulk} factors consistent for the reductive dechlorination of chlorinated ethenes (36-37). Because accurate ε_{bulk} factors are required for the quantification of biodegradation extent, it is important to ensure that the measured variability of ε_{bulk} values is reproducible in independent experiments. Furthermore, by determining ε_{bulk} values for a greater number of bacterial isolates, patterns in fractionation may emerge, potentially explaining the observed variability. In Chapter 6, ε_{bulk} factors for chlorinated ethene dechlorination by *Dhc* pure and mixed cultures are determined and the possible reasons for variation in ε_{bulk} factors are discussed. Results from experiments determining ε_{bulk} factors for the dichloroelimination of 1,2-D in two unique cultures are presented in Chapter 7.

1.2.3 Objective 3: Determine whether U(VI) Reduction is a Universal Trait of *Desulfitobacterium* and Identify the Reduced Product

One of the results of the experiments described in Chapter 4 was the isolation of the novel PCE-dechlorinating *Desulfitobacterium hafniense* strain JH1. Because gram positive *Desulfitobacterium* are commonly present in subsurface environments, are metabolically versatile, and have been found at a number of U(VI) contaminated sites (41-43, 49), U(VI) reduction was investigated in strain JH1 as well as a number of other *Desulfitobacterium* isolates. Whereas U(VI) is generally reduced to insoluble UO_2, recent reports have demonstrated that other U(IV) products may also be formed (45-47). Characteristics of the U(IV) product determine the stability and mobility of the reduced uranium, which is critical for uranium subsurface fate. Therefore, the U(IV) precipitate produced by the *Desulfitobacterium* isolates was also identified. The results from U(VI) reduction experiments conducted with *Desulfitobacterium* spp. and the identity of the reduced product are presented in Chapter 8.

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CHAPTER 2

LITERATURE REVIEW

2.1 Chapter Overview

This chapter contains a brief review of the relevant literature pertaining to chlorinated solvent and uranium contamination and remediation. The first section details the causes and scope of chlorinated solvent contamination. The next section describes the microbial populations, molecular mechanisms, and metabolic pathways involved in the detoxification of chlorinated compounds. The third section includes discussions of the application of bioremediation at contaminated sites, the potential for coupling bioremediation to thermal treatment, and the tools available for monitoring bioremediation. The environmental scope of uranium contamination and the microbial immobilization of uranium are considered in the final two sections. The available literature is further reviewed in Chapters 3 - 8.

2.2 Environmental Scope of Chlorinated Solvent Contamination

Many toxic chlorinated compounds are produced naturally (1-2); however, long-term anthropogenic production of chlorinated compounds (2) coupled to inappropriate disposal practices has resulted in widespread chlorinated compound contamination (3). Chlorinated alkenes such as the chlorinated ethenes tetrachloroethene (PCE) and trichloroethene (TCE) are solvents used for dry cleaning and degreasing applications (3).

In the environment, PCE and TCE are degraded to other chlorinated alkenes, such as *cis*-1,2-dichloreothene (*cis*-DCE), *trans*-1,2-dichloroethene (*trans*-DCE), and vinyl chloride (VC) (2, 4-5). VC is also produced anthropogenically as an intermediate in the production of polyvinyl chloride (PVC) (6). Chlorinated alkanes, including chlorinated ethanes and chlorinated propanes, have also been used for metal degreasing, are produced as intermediates in PVC production, and have even been used as soil fumigants (6-7). 1,2-D is also generated during the production of other chlorinated compounds including carbon tetrachloride and PCE (6, 8).

Because PCE and TCE are toxic and suspected human carcinogens (7), they are regulated in drinking water at a maximum contaminant level (MCL) of 5 parts per billion (ppb) (Table 2.1) (U.S. Environmental Protection Agency (EPA); http://www.epa.gov/safewater/contaminants). The drinking water MCLs for *cis*- and *trans*-DCE are 70 and 100 ppb, respectively (EPA; http://www.epa.gov/safewater/contaminants). VC is a proven human carcinogen (7) and is therefore regulated in drinking water at 2 ppb (EPA; http://www.epa.gov/safewater/contaminants). 1,2-DCA and 1,2-D are also toxic (6, 9) and suspected to cause cancer and are regulated in drinking water at 5 ppb (EPA; http://www .epa.gov/safewater/contaminants).

In the U.S. alone, over 700 sites are contaminated with PCE and/or TCE according to the Comprehensive Environmental Response, Compensation and Liability Information System (CERCLIS) database (http://cfpub.epa.gov/supercpad/cursites /srchsites.cfm). In fact, in groundwater at National Priorities List (NPL) sites, TCE is the most frequently detected chemical contaminant (10). Chlorinated alkanes such as 1,2-DCA and 1,2-D are found at over 353 and 118 U.S. sites, respectively (CERCLIS), and

chlorinated alkanes are among the most frequently detected contaminants in both the U.S. and in Europe (6, 9).

Chlorinated compounds with low aqueous solubilities and high densities, such as PCE and TCE (Table 2.1) may exist as dense, non-aqueous phase liquids (DNAPLs) in the subsurface (11-13). DNAPLs form when a chlorinated compound migrates through the vadose zone to the saturated zone and displaces groundwater to continuously move downward until it encounters a low permeability layer (14). Once a low permeability layer is encountered, the chlorinated compound will pool (15-16). This DNAPL pool acts as a long-term source of groundwater contamination (11-12).

Table 2.1 Summary of physical properties and MCLs for select chlorinated alkenes and chlorinated alkanes (adapted from Amos (17)).

Compound	Density[a] (g/mL)	Aqueous Solubility (mg/L)	K_h[g] -	MCL[h] (ppb)
Chlorinated Alkenes				
PCE	1.63	250[c]	0.69	5
TCE	1.46	1100[d]	0.40	5
cis-DCE	1.25	3500[a]	0.17	70
trans-DCE	1.26	6300[a]	0.45	100
VC	NA[b]	1100[e]	1.00	2
Chlorinated Alkanes				
1,2-D	1.16	2700[f]	0.12	5
1,2-DCA	1.25	7600[e]	0.05	5

[a] Densities and some aqueous solubilities are from (18).
[b] NA indicates that values are not applicable as the compound is a gas at ambient temperatures.
[c] Aqueous solubility is from (19).
[d] Aqueous solubility is from (20).
[e] Aqueous solubilities are from (21).
[f] Aqueous solubility is from (22).
[g] K_h, dimensionless Henry's law constants are calculated for 25°C based on values presented in (23).
[h] MCL values are from the EPA; http://www.epa.gov/safewater/contaminants.

2.3 Microbial Detoxification of Chlorinated Compounds

Under aerobic conditions, some chlorinated compounds support microbial growth as the sole source of carbon and energy. For example, the transformation of VC to CO_2 and inorganic chloride supports the growth of bacteria in the *Mycobacterium*, *Nocardioides*, *Polaromonas*, *Pseudomonas*, and *Rhodococcus* genera (24-28). Furthermore, a beta-Proteobacterium similar to *Polaromonas vacuolata*, grows via the transformation of *cis*-DCE to CO_2 (28). Conversely, 1,2-D has been shown to be recalcitrant under aerobic conditions (29) and there are no known respiratory aerobic pathways for the degradation of PCE or TCE (11).

Under anaerobic conditions, a range of microbes have been reported to couple growth with the reductive dechlorination of PCE and TCE, including bacteria within the *Clostridium*, *Dehalobacter*, *Dehalococcoides* (*Dhc*), *Desulfitobacterium*, *Desulfuromonas*, *Geobacter*, and *Sulfurospirillum* genera (30-36). The only organisms capable of deriving energy from the dechlorination of DCE isomers and VC are *Dhc*, although not all *Dhc* populations are capable of metabolic DCE and VC dechlorination (Table 2.2) (4, 37). Metabolic reductive dechlorination of chlorinated ethenes requires multiple, stepwise reactions (7) and reaction specifics are discussed in greater detail in section 2.3.1. Growth-linked dechlorination of 1,2-D is catalyzed by *Dehalobacter* populations, *Dhc* populations, *Dehalogenimonas lykanthroporepellens* strains, and *Desulfitobacterium dichloroeliminans* strain DCA1 (38-41). In the majority of cases, 1,2-D has been shown to be converted directly to propene via a dichloroelimination reaction. Dichloroelimination reactions are described in greater detail in section 2.3.2.

Under both aerobic and anaerobic conditions, cometabolic (non growth-yielding) reactions may also contribute to chlorinated compound degradation (10-11). Monoxygenases, which are found in methanotrophic bacteria, have been demonstrated to catalyze oxidation of TCE, *cis*-DCE, VC, and 1,2-D in the presence of O_2 and a co-substrate (10, 42-43). Under anaerobic conditions, chlorinated ethenes may be cometabolized by *Dhc* in the presence of a metabolic electron donor (Table 2.2) (4, 36-37). Because cometabolic processes are slow and rely on the presence of a co-substrate, metabolic processes are preferred for bioremediation (3, 44).

2.3.1 Molecular Mechanisms of Chlorinated Solvent Transformation

The hypothetical pathway for VC biodegradation in *Nocardioides* sp. strain JS614 is shown in Figure 2.1 (45). VC is initially oxidized to chlorooxirane by alkene monooxygenase (likely a soluble di-iron monooxygenase) and chlorooxirane is metabolized further by epoxyalkane:coenzyme M transferase (45-46). After the addition of coenzyme M, HCl is spontaneously cleaved and a coenzyme A is bound to the residual compound. Following the cleavage of coenzyme M, the molecule is metabolized in the TCA cycle (45). In both *Nocardioides* sp. strain JS614 and *Mycobacterium* strain JS60, the vinyl chloride monooxygenase is encoded by the *etnABCD* genes and the epoxyalkane:coenzyme M transferase is encoded by the *etnE* gene (45, 47). Interestingly, experimental results have demonstrated definitively that the *etnABDCD* and *etnE* genes are carried on a plasmid in strain JS614 and are likely also carried on a plasmid in strain JS60 (45, 47).

Genes encoding for the proteins that catalyze the reductive dechlorination of halogenated compounds have been termed reductive dehalogenases (RDases). *Dhc* RDases that catalyze the dechlorination of TCE, DCE isomers, and VC have been identified using both proteomic and molecular techniques (48-50). Specifically, the *bvcA* gene in strain BAV1 encodes BvcA, which catalyzes VC dechlorination (48), the *tceA* gene in strain 195 and strain FL2 encodes TceA, which catalyzes TCE and DCE dechlorination (37, 49), and the *vcrA* gene in strain GT and strain VS encodes VcrA, which catalyzes DCE and VC dechlorination (Table 2.2) (50-51). Most identified RDases are corrinoid-dependent and contain two iron-sulfur clusters (52). Dehalogenation reactions are linked to electron transport-coupled phosphorylation by anchoring proteins that ensure that RDases are closely associated with the cytoplasmic membrane (52-53). No RDases catalyzing the dichloroelimination of 1,2-D have been identified.

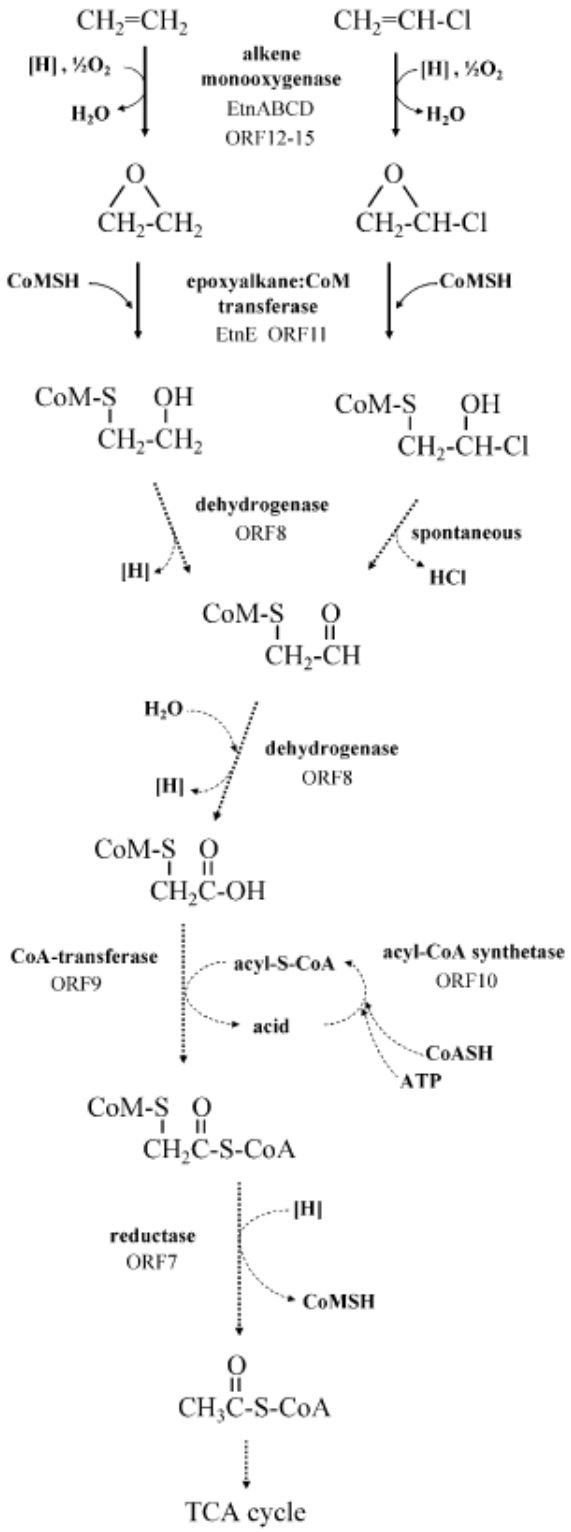

Figure 2.1 The hypothetical pathway of ethene and VC biodegradation in *Nocardioides* sp. JS614 (45).

2.3.2 Reductive Dechlorination of Chlorinated Ethenes

Reductive dechlorination of chlorinated ethenes involves the replacement of a chlorine atom with a hydrogen atom (3). Therefore, in the case of chlorinated ethenes, reductive dechlorination reactions result in the stepwise transformation of PCE to TCE, DCE, VC, and nontoxic ethene (Figure 2.2) (3). For each reductive dechlorination reaction, two electrons are transferred from an electron donor to the chlorinated ethene, which serves as the electron acceptor and, as such, is reduced (3). One proton and one chloride ion are released in each dechlorination step. A variety of electron donors are used by bacteria that reduce PCE and TCE to DCE including acetate, hydrogen, and pyruvate (34, 54). *Dhc* only couple the reduction of chlorinated ethenes to the oxidation of hydrogen (3).

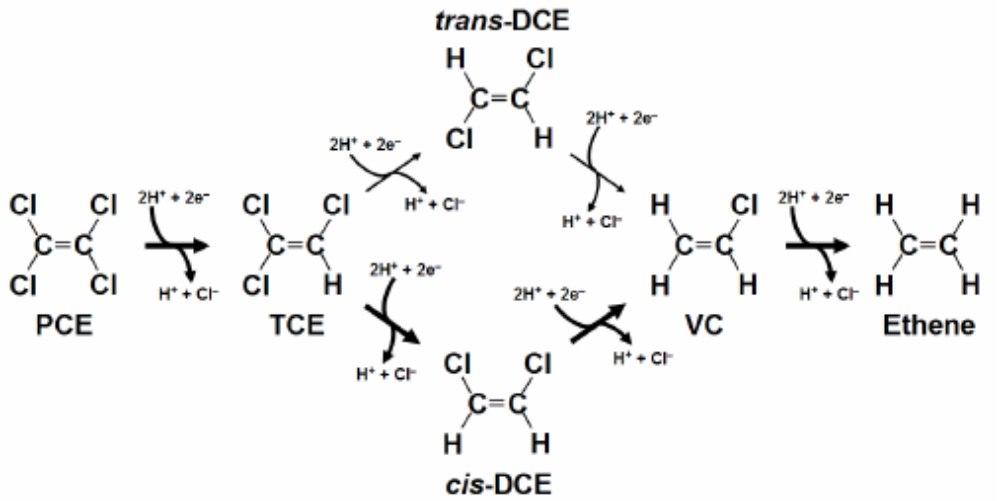

Figure 2.2 Stepwise reductive dechlorination of chlorinated ethenes to ethene (3).

Table 2.2 Properties of *Dhc* strains (adapted from Löffler and Edwards (3)).

***Dhc* strain**	**Metabolic Electron Acceptors**	**Cometabolized Chlorinated Ethenes**	**Biomarker RDase[a]**	**Reference**
Strain 195	PCE, TCE, *cis*- and 1,1-DCE, 1,2-DCA	*trans*-DCE, VC	*tceA*	(36, 55)
Strain BAV1	*cis*-, *trans*, and 1,1-DCE, VC, 1,2-DCA	PCE, TCE	*bvcA*	(4)
Strain FL2	TCE, *cis*- and *trans*-DCE	PCE, VC	*tceA*	(37)
Strain GT	TCE, *cis*- and 1,1-DCE, VC	None	*vcrA*	(51)
Strain KS	1,2-D	None	ND[b]	(39)
Strain MB	PCE, TCE	None	*dceA1*	(5)
Strain RC	1,2-D	None	ND	(39)
Strain VS	TCE, *cis*- and 1,1-DCE, VC	ND	*vcrA*	(50, 56)

[a] RDases are reductive dehalogenases.
[b] ND indicates that either chlorinated ethene cometabolism has not been investigated or that the RDase has not been identified.

2.3.3 Dichloroelimination of Chlorinated Alkanes

Chlorinated alkanes that have two chlorine substituents on adjacent, single bonded carbon atoms, such as 1,2-DCA and 1,2-D (Figure 2.3), may undergo dichloroeliminations, or vicinal reduction reactions (7). In a dichloroelimination reaction, both carbon-chlorine bonds are broken and a double bond is formed between the adjacent carbon atoms without the formation of intermediates as shown in Figure 2.3 for the transformation of 1,2-D to nontoxic propene (7). Similarly, 1,2-DCA undergoes dichloroelimination to form nontoxic ethene (4, 55). In dichloroelimination reactions, two electrons are transferred from an electron donor (e.g., hydrogen or formate) to the chlorinated alkane and two protons and two chloride ions are released. Although dichloroelimination reactions proceed without the measurable production of intermediates, it is unknown if the rate-limiting step of the reaction involves one or two carbon atoms and is therefore a stepwise or concerted reaction, respectively (57). Whereas *Dhc*, *Dehalobacter*, and *Dehalogenimonas lykanthroporepellens* populations are only known to use hydrogen as an electron acceptor (4, 32, 58), *Desufitobacterium* strain DCA1 couples the reduction of 1,2-D and 1,2-DCA to formate, lactate, and hydrogen oxidation (41).

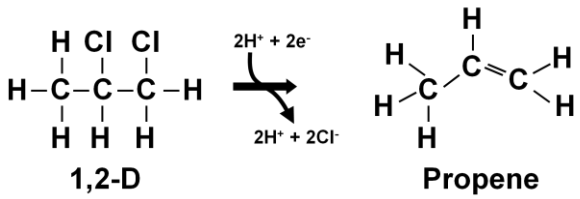

Figure 2.3 Dichloroelimination of 1,2-D to propene (adapted from Löffler et al. (7)).

2.4 Bioremediation of Chlorinated Solvent-Contaminated Sites

Bioremediation is the exploitation of biologically catalyzed reactions for the clean-up or containment of contamination. Bioremediation was first demonstrated as a viable remediation strategy in the 1970s and ‘80s for the clean-up of petroleum hydrocarbon-contaminated sites (i.e., oil spills) (3, 59). Since then, the accepted uses of bioremediation have expanded to include the clean-up of subsurface environments contaminated with both chlorinated alkenes and chlorinated alkanes. In this section, studies demonstrating the effectiveness of in situ bioremediation are reviewed and the potential risks and benefits of coupling bioremediation with thermal treatment are discussed. The section concludes with a description of the current tools available for the monitoring of reductive dechlorination reactions.

2.4.1 Biostimulation and Bioaugmentation for In Situ Remediation

A number of studies have demonstrated the effectiveness of reductive dechlorination for the remediation of chlorinated ethene plumes (60-63). In all cases, for complete reductive dechlorination to ethene to occur, *Dhc* populations capable of VC dechlorination had to be present and active (3). Interestingly, *Dhc* are not naturally present at all chlorinated ethene-contaminated sites (3). In these cases, mixed cultures containing *Dhc* may be amended to the subsurface in a process termed bioaugmentation (3). In order to be viable and active, native or introduced *Dhc* require available electron donor (64). Anoxic conditions are also vital for successful bioremediation as *Dhc* are obligate anaerobes that may be inhibited after brief exposure to oxic conditions (65).

Electron donor is amended to the subsurface to fuel dechlorination and to stimulate the production of reducing conditions in a process termed biostimulation (3). Although *Dhc* are strict hydrogenotrophs (3), a variety of compounds ranging from lactate to emulsified vegetable oil to chitin are used to increase hydrogen flux and to fuel reductive dechlorination in situ (3, 66-67).

Reductive dechlorination of 1,2-D is catalyzed by a number of organisms in addition to *Dhc* populations (39-41, 58), but the same general techniques employed for chlorinated ethene bioremediation (i.e., bioaugmentation, biostimulation) may also be applied for bioremediation of 1,2-D contaminated sites. Furthermore, according to the CERCLIS database (http://cfpub.epa.gov/supercpad/cursites/srchsites. cfm), chlorinated ethenes are co-contaminants at most 1,2-D contaminated sites. Therefore, bioremediation of 1,2-D may simply include the additional step of ensuring that native or introduced populations are capable of 1,2-D dechlorination.

2.4.2 Bioremediation Coupled to Thermal Treatment

Whereas bioremediation has been shown to enhance the rate of DNAPL dissolution as compared to abiotic dissolution alone (68), aggressive source zone techniques, including co-solvent or surfactant flooding, chemical oxidation, and thermal treatment, remove or destroy DNAPL mass more rapidly than bioremediation (11, 64). Yet, these aggressive techniques do not typically remove 100% of contaminant mass (11) and therefore, interest has developed in coupling aggressive source zone remediation techniques with bioremediation (11, 64, 69). Thermal treatment coupled to bioremediation has been suggested to be a promising strategy for remediation of

chlorinated ethene-contaminated sites because i) thermal treatment does not introduce potentially inhibitory compounds (e.g, solvents, surfactants, and oxidants) to the subsurface, ii) thermal treatment releases organic carbon from the subsurface matrix, which may provide electron donors for dechlorinators (69), and iii) elevated temperatures may enhance reductive dechlorination activity (69-70). However, there are also risks of coupling thermal treatment with bioremediation. Specifically, the released organic carbon may fuel competing terminal electron accepting processes (e.g., methanogenesis) (71) and complete dechlorination to ethene has never been reported to occur at typical source zone temperatures during thermal treatment (up to or even exceeding 100°C (72)) (70).

In a previous study, Friis et al. demonstrated that less methane was produced in microcosms that were previously heated at 100°C for 10 days than in unheated microcosms, even when microcosms were bioaugmented with a methanogenic PCE-to-ethene dechlorinating culture (71). These results imply that the organic carbon released during heating did not fuel increased competition for electron donor. Furthermore, in this previous study, complete dechlorination of TCE to ethene occurred in two out of three previously heated microcosms both with and without electron donor addition. Conversely, although complete dechlorination to ethene occurred in all unheated and biostimulated microcosms, complete dechlorination occurred in only one of three unheated microcosms that were not biostimulated. These results suggest that thermal treatment stimulated the release of organic carbon, making biostimulation unnecessary for complete dechlorination to ethene (71); however, because microcosms were constructed from materials from only one site and initial hydrogen concentrations in the

majority of microcosms were at least 3 orders-of-magnitude greater than concentrations typically found in groundwater (71, 73), the applicability of these results to diverse contaminated sites is unclear.

Temperature optima have been identified for reductive dechlorination of chlorinated ethenes by mixed and pure cultures (70). Because distinct microbes are involved in individual reductive dechlorination steps, some steps of the dechlorination reaction occur at different maximum temperatures (Table 2.3). Specifically, whereas one study has reported that reductive dechlorination of PCE to *cis*-DCE occurs at temperatures of up to 65°C (74), metabolic reductive dechlorination of VC has only been reported to occur at temperatures up to 30°C (75-76). Due to the mesophilic temperatures required for complete dechlorination to ethene, ethene production is not expected to occur in the source zone during thermal treatment (70). Even so, because optimal temperatures for dechlorination reactions are higher than the temperature of most aquifers, dechlorination activity may actually be enhanced in portions of the temperature gradient in the perimeter of the source zone. Furthermore, dechlorination may occur in the source zone during cool-down. Few studies have examined the potential for dechlorinators to recover activity following exposure to elevated temperatures, although spore-forming dechlorinators such as *Clostridium bifermentans* strain DPH-1 are likely to recover from exposure to elevated temperatures.

2.4.3 Monitoring Bioremediation

In order to demonstrate that decreases in contaminant concentrations are due to in situ microbial reductive dechlorination rather than the effects of dispersion, sorption, or volatilization, multiple lines of evidence are required (77). Because the end-products of

degradation of chlorinated ethenes and 1,2-D are known and quantifiable, metabolite analysis can be applied to provide evidence for biodegradation (77-79). For example, at sites contaminated with TCE, the detection of increasing concentrations of *cis*-DCE, VC, and ethene support the conclusion that in situ dechlorination is occurring (61-62, 80). However, the usefulness of metabolite analysis is limited when sites have co-contaminants (77, 81). For example, at a site contaminated with both TCE and 1,2-DCA, metabolite analysis alone could not be used to determine if the ethene was derived from the TCE or the 1,2-DCA (82-83).

Molecular analyses of nucleic acids (DNA and RNA) may provide an additional line of evidence for in situ biodegradation (3, 61-62). Many organisms involved in chlorinated ethene and 1,2-D dechlorination have been identified and molecular assays targeting the 16S rRNA genes of these populations have been designed (76, 86-87). Due to limitations of the specificity of 16S rRNA gene targets, assays have also been designed that target the specific RDase genes that code for enzyme systems catalyzing dechlorination reactions (88-89). Molecular tools for the assessment of in situ dechlorination are further discussed in section 2.4.3.1.

Table 2.3 Temperatures with maximum dechlorination rates (adapted from Friis et al. (70)).

Culture	Metabolic Substrate Range	Temperature with Maximum Rate (°C)	Reference
Dehalobacter restrictus	PCE to *cis*-DCE	25-30	(32)
Desulfitobacterium spp.	PCE to *cis*-DCE	34-38	(31, 54, 84)
Dhc strain 195	PCE to VC	35	(55)
Dhc strain BAV1	DCEs to ethene	22-30	(4)
Dhc strain FL2	TCE to VC	20-30	(37)
Geobacter lovleyi strain SZ	PCE to *cis*-DCE	35	(51)
KB1	TCE to ethene	15-30	(70)
Culture from Contaminated Sediments	PCE to *cis*-DCE	60-65	(74)
Culture from Contaminated Soil	PCE to VC	35	(85)
Sulfurospirillum multivorans	PCE to *cis*-DCE	30	(33)

Although metabolite analysis and molecular techniques can provide evidence for in situ dechlorination, compound specific stable isotope analysis (CSIA) can actually be used to estimate in situ dechlorination extent (90). CSIA takes advantage of the slower rate of degradation of organic compounds containing heavy isotopes as compared to compounds containing only light isotopes (57). Because isotope fractionation only occurs when a compound undergoes degradation and does not occur due to sorption, volatilization, or dilution, CSIA can be used to quantify the fraction of the disappearance of a compound that is actually due to compound degradation (83). CSIA has been used to demonstrate the in situ dechlorination of chlorinated ethanes (82-83) and chlorinated ethenes (82-83, 91-94). A further discussion of CSIA is in section 2.4.3.2.

2.4.3.1 Molecular Techniques

Dhc are vital for the complete dechlorination of PCE or TCE to ethene (3) and molecular tools that quantify the 16S rRNA gene of *Dhc* are available (76, 95). Many *Dhc* 16S rRNA genes are indistinguishable due to their high degree of similarity (3, 89, 95), but not all *Dhc* populations are capable of metabolic dechlorination to ethene (Table 2.2). For example, the 16S rRNA gene of *Dhc* strain FL2 is 100% identical to that of strain CBDB1, which has not been reported to dechlorinate any chlorinated ethenes (37, 96). Therefore, additional molecular tools target specific *Dhc* RDase genes (89, 95). Assays have been developed for the quantification of the *bvcA* gene (48), the *tceA* gene (37, 49), and the *vcrA* gene (Table 2.2) (50-51). Molecular tools targeting the genera that contain 1,2-D dechlorinating populations (*Dehalobacter*, *Dehalogenimonas*,

Desulfitobacterium, and *Dhc*) also exist (87, 97), but not all isolates within these genera demonstrate 1,2-D dechlorination activity (Table 2.2) (4, 41). Because the RDase(s) catalyzing this reaction have not been identified, no molecular techniques exist for specifically monitoring 1,2-D dechlorinators.

Molecular techniques generally rely on the polymerase chain reaction (PCR), in which specific genes are targeted by primers. Previously, PCR was mainly used for qualitative analysis of gene presence or absence, but, currently, assays rely on quantitative real-time PCR (qPCR) (89). In qPCR, the target gene is enumerated, allowing for the number of gene copies in a sample to be determined (89). Generally, qPCR is used to enumerate gene copies in a DNA sample and many studies have demonstrated that temporal changes in *Dhc* gene abundances correlate with dechlorination activity (61-62, 68, 76, 80, 88). Despite the demonstrated correlation between *Dhc* growth and dechlorination activity, the detection of *Dhc* 16S rRNA genes or even specific RDase genes in DNA does not indicate that *Dhc* cells are active (80, 98). Therefore, interest has developed in collecting RNA samples, performing reverse transcription (transcribing RNA to complementary DNA (cDNA)), and quantifying gene copies in cDNA (which is equivalent to the quantity of gene transcripts) (80, 98). Presumably, only active, viable cells produce gene transcripts, and therefore, quantification of gene transcripts is considered to be a more reliable indicator of metabolic state and activity than quantification of gene copies alone (80, 98). In fact, several studies have demonstrated that RDase gene transcript abundances correlate with dechlorination activity during active dechlorination (80, 98-104). However, one recent study demonstrated that when *Dhc* are inhibited by oxygen, gene transcripts are still

detectable and may even be present at abundances per cell similar to or greater than abundances in actively dechlorinating cultures (98). These results suggest that gene transcript abundances may not accurately represent dechlorination activity under inhibitory conditions.

2.4.3.2 Compound Specific Stable Isotope Analysis

Most chlorinated ethenes and 1,2-D contain carbon, chlorine, and hydrogen, all of which have multiple naturally occurring isotopes, (^{12}C and ^{13}C, ^{35}Cl and ^{37}Cl, and ^{1}H and ^{2}H) (90). CSIA is used to quantify the effect of heavy isotopes on bond cleavage (57, 90) and therefore, since only carbon-chlorine bonds are broken during the reductive dechlorination of chlorinated ethenes and 1,2-D (Figure 2.2 and Figure 2.3), only analysis of carbon or chlorine isotopes is applicable. Although interest is increasing in measuring chlorine isotopes (105-106), due to analytical challenges, CSIA for the quantification of chlorinated compound degradation generally relies on carbon isotope measurements (82-83, 91-94).

Isotope effects are generally quantified according to the Rayleigh model (57, 107):

$$ln((1000+ \delta^{13}C_0 + \Delta\delta^{13}C)/(1000+ \delta^{13}C_0)) = (\varepsilon_{bulk}/1000)ln(f) \quad (2.1)$$

where $\delta^{13}C_0$ is the carbon isotope composition of the parent compound at time zero, $\Delta\delta^{13}C$ is the change in the carbon isotope composition from time zero to time t, ε_{bulk} is the bulk carbon isotope enrichment factor, and f is the molar fraction of the parent compound remaining at time t. Once $\delta^{13}C_0$ and the ε_{bulk} factor are known, the in situ biodegradation extent can be estimated (90).

Whereas no studies have assessed the isotope effects of 1,2-D degradation, a number of studies have determined ε_{bulk} factors for the reductive dechlorination of chlorinated ethenes by pure and mixed cultures (82-83, 91-94, 107-109). However, reported ε_{bulk} factors vary between studies even for the reductive dechlorination of *cis*-DCE and VC, which are only known to be dechlorinated by *Dhc* (109). In the two *Dhc* pure cultures for which ε_{bulk} factors have been derived, ε_{bulk} factors for *cis*-DCE dechlorination varied from -21.1 ± 1.8 to -16.4 ± 1.4‰ by strain 195 and strain BAV1, respectively (109). This variation in ε_{bulk} factors may be caused by the different RDases that catalyze *cis*-DCE dechlorination in these strains (TceA in strain 195 and an unidentified RDase in strain BAV1 (48-49)). Therefore, it is possible that the specific RDase catalyzing the dechlorination reaction plays an important role in determining fractionation extent. Because ε_{bulk} factors have not been derived for unique *Dhc* strains that possess the same RDase, it is currently impossible to determine whether ε_{bulk} factors for dechlorination by *Dhc* are RDase-specific.

Interestingly, whereas ε_{bulk} factors for multiple dechlorination reactions have been determined (109), few studies have compared isotope effects between different molecules for identical reactions catalyzed by similar microbial populations. For example, *Dhc* catalyze both dichloroelimination of 1,2-D and 1,2-DCA, but no studies have determined whether the isotope effects associated with 1,2-D dichloroelimination are consistent with those measured for 1,2-DCA dichloroelimination. By calculating apparent kinetic isotope effect (AKIE) values, the isotope effects associated with bond cleavages in different molecules can be directly compared. AKIE values are normalized for the

presence of non-reactive and indistinguishable reactive sites and are calculated according to (57):

$$AKIE = 1/(1+(z * \varepsilon_{reactive}/1000)) \quad (2.2)$$

where z, the number of indistinguishable reactive sites, is a correction for the effects of intramolecular competition and $\varepsilon_{reactive}$ is the reactive position-specific enrichment factor, which is determined according to (57):

$$ln((1000+\delta^{13}C_0 +(n/x)\,\Delta\delta^{13}C)/(1000+\delta^{13}C_0)) = (\varepsilon_{reactive}/1000)ln(f) \quad (2.3)$$

where n is the number of carbon atoms in the molecule and x is the number of carbon atoms in the reactive position.

2.5 Environmental Scope of Uranium Contamination

Uranium is the 49th most abundant element on the Earth's crust and therefore, may naturally be found in groundwater due to the erosion of uranium deposits (110). However, uranium contamination also has anthropogenic sources such as the processing of uranium for power and weapons production (111-112). Currently, over 100 U.S. Department of Energy (DOE) sites report uranium contamination (112). Furthermore, according to the CERCLIS database, sites ranging from a sewage treatment plant in Illinois to a landfill in Pennsylvania to a chemical plant in New Jersey are also contaminated with uranium (http://cfpub.epa.gov/supercpad/cursites/srchsites.cfm).

Naturally occurring uranium has three primary isotopes, ^{234}U, ^{235}U, and ^{238}U, which comprise 0.005, 0.72, and 99.27%, of total uranium in the environment, respectively (111). ^{238}U has the longest half-life, or is the least radioactive, of the

primary isotopes and therefore, depleted uranium is enriched in ^{238}U (113). Although all uranium isotopes are radioactive, naturally occurring and depleted uranium are primarily chemical rather than radiological hazards (111, 113). Uranium exposure is most likely to affect the kidneys, although inhalation of uranium may also affect the lungs (113). The MCL for uranium, as mandated by the EPA, is 30 ppb (EPA; http://www.epa.gov/safewater/contaminants).

In addition to occurring at varying isotope compositions, uranium also naturally occurs in both the +6 and +4 oxidation states (112). Including both oxidation states, uranium has been shown to exist as over 40 different dissolved species and as a part of at least 30 solid phases (114). Even in water alone, uranium chemistry is complex as shown in the Eh (redox potential)-pH diagram in Figure 2.4 (114). In the presence of carbonate, calcium, phosphate, and humic substances, even more uranium species may occur (Figure 2.5) (114). Uranium speciation is complex and reliant on pH, Eh, temperature, and ligand concentration, but in oxic waters at pH 5.0 to 8.5 with limited calcium carbonate, uranium generally exists as $(UO_2)_2(OH)_3CO_3^-$ and $UO_2(OH)_3^-$ (111-112). In the presence of higher carbonate concentrations, uranium-carbonate complexes dominate (112, 115). Solid uranium species are less likely to migrate with groundwater flow and spread uranium contamination (112, 116). The main bioremediation approach for uranium contamination is uranium immobilization via the production of insoluble solid phases (112).

2.6 Microbial Immobilization of Uranium

Uranium immobilization can occur via the complexation of uranium with orthophosphates or via changes in the oxidation state of uranium (112, 116). Recent reports have demonstrated that some bacteria possessing phosphatases fuel uranium complexation with phosphate by hydrolyzing organophosphates, which releases PO_4^{3-} (116-118). Because it is unlikely that in situ quantities of organophosphates are adequate to fuel significant immobilization of U(VI), studies have suggested employing biostimulation with organophosphates to promote U(VI) immobilization (118).

The bacterial reduction of U(VI) to U(IV) generally yields the insoluble uraninite mineral (UO_2) (112). Interestingly, U(VI) has been shown to serve as the terminal electron acceptor in the metabolism of some bacterial (119-122). UO_2 and bacteria capable of U(VI) reduction have been identified in subsurface environments, suggesting that U(VI) reduction is environmentally relevant (123-124); however, U(VI) reduction requires anoxic conditions and UO_2 is oxidized, and therefore re-solubilized, by exposure to oxygen, nitrite or Fe(II) oxyhydroxides (125-128). Immobilization of uranium via microbial reduction is further discussed in sections 2.6.1 and 2.6.2.

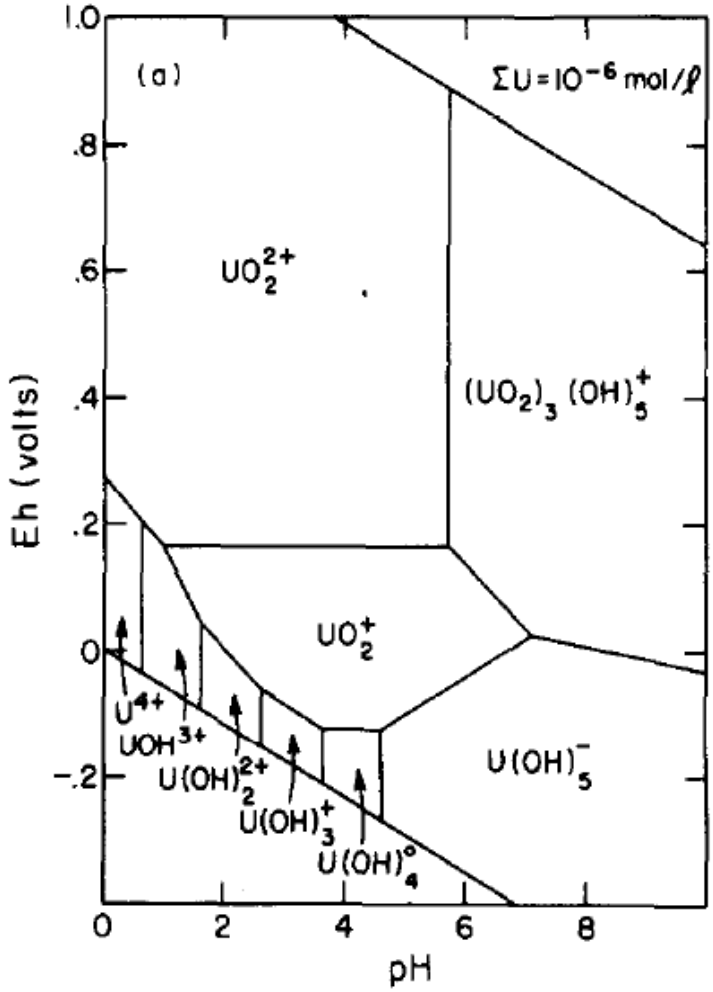

Figure 2.4 Eh-pH diagram of uranium speciation. Calculations were performed assuming a total uranium concentration of 10^{-6} M (114).

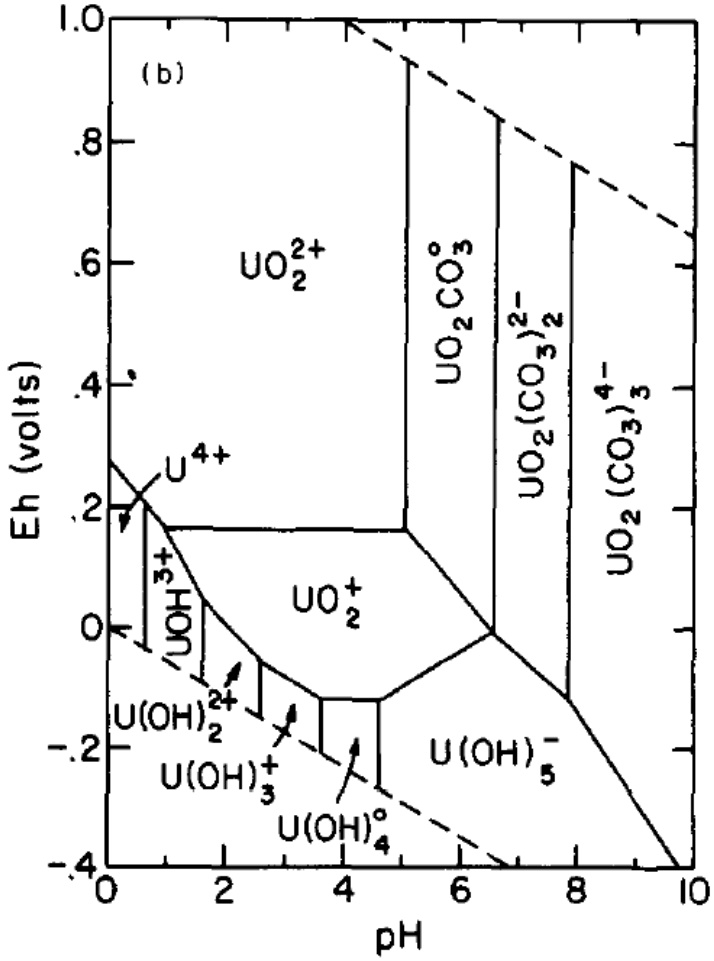

Figure 2.5 Eh-pH diagram of uranium speciation in the presence of CO_2. Calculations were performed assuming a total uranium concentration of 10^{-6} M and 10^{-2} atm CO_2 (114).

2.6.1 Bacteria Capable of U(VI) Reduction

Bacterial isolates capable of U(VI) reduction are phylogentically diverse and are found in 17 unique genera (112, 129). The majority of isolates are within the Clostrida, delta-Proteobacteria, and gamma-Proteobacteria classes (112). Within the Clostridia class, isolates from the *Clostridium*, *Desulfitobacterium*, *Desulfosporosinus*, *Desulfotomaculum*, *Thermoanaerobacter*, *Thermoterrabacterium*, and *Veillonella* genera reduce U(VI) (121, 130-134). U(VI) reducing isolates within the delta-Proteobacteria include populations in the *Anaeromyxobacter*, *Desulfomicrobium*, *Desulfovibrio*, and *Geobacter* genera (119-120, 135-136). Gamma-Proteobacteria within the *Pseudomonas*, *Salmonella*, and *Shewanella* genera have also been reported to reduce U(VI) (120, 137-138). Interestingly, *Pyrobaculum islandicum*, a thermophillic Archaea, also reduces U(VI) (139).

Previous studies have focused on U(VI) reduction by gram negative Proteobacteria, even though many gram positive isolates have also been shown to reduce U(VI) (112) and both U(VI) reducing gram positive and gram negative isolates have been found at uranium-contaminated sites (140-143). Many uranium-contaminated sites, including the U.S. Department of Energy (DOE) Integrated Field-Scale Subsurface Research Challenge (IFC) site, are both uranium-contaminated and acidic (pH 4 to 5). Interestingly, one study has suggested that gram positive rather than gram negative organisms are better equipped for the acidic conditions prevalent at this site (143). Knowledge of in situ U(VI) reduction is limited by a lack of studies regarding the role of gram positive bacteria.

Many studies have reported U(VI) reduction by a single isolate, but have not determined whether U(VI) reduction is a common trait among related organisms (e.g., isolates within a genus) (121, 129, 136). For example, Shelobolina et al., reported that *Desulfitobacterium hafniense* strain G2 is capable of U(VI) reduction but did not investigate U(VI) reduction in any other *Desulfitobacterium* isolates (129), even though *Desulfitobacterium* have been identified at multiple U(VI)-contaminated sites (140, 142-143).

In most studies, U(VI) reduction is demonstrated only by resting cell assays (Table 2.4) (125, 129, 144-147). Standard resting cell assays are performed by growing cells in complex medium, collecting cells via centrifugation, washing cells, and suspending washed cells in fresh buffer amended with U(VI) (148). U(VI) reduction assays are typically conducted with cell titers up to or even greater than 10^9 cells/mL (146, 148). Because of the elevated cell titers, the U(VI) reduction rates obtained in such assays are not environmentally relevant. For example, in a resting cell experiment conducted with 2 x 10^9 *Anaeromyxobacter dehalogenans* strain 2CP-C cells/mL, 250 µM U(VI) was reduced in under 25 hours (146). Conversely, in an experiment conducted with a strain 2CP-C titer of 10^6 cells/mL, 200 µM U(VI) was reduced in 10 days (119). Experiments conducted under environmentally relevant conditions are required to accurately estimate U(VI) reduction rates.

2.6.2 Molecular Mechanism of U(VI) Reduction

The tetraheme cytochrome c_3 and a periplasmic hydrogenase have been implicated in U(VI) reduction in *Desulfovibrio vulgaris* (149); however, U(VI) reduction

occurred even in *Desulfovibrio* mutants without the tetraheme cytochrome, demonstrating that other proteins capable of U(VI) reduction are also present (150). In *Shewanella* spp., experimental results also suggested that cytochromes are involved in U(VI) reduction and that multiple U(VI) reductases are present (112). Yet, no dedicated U(VI) reductases have been identified in any U(VI) reducer and the precise mechanism of U(VI) reduction remains unknown (112). Furthermore, whereas U(VI) reduction to U(IV) requires the transfer of two electrons to U(VI), evidence suggests that *Geobacter sulfurreducens* only reduces U(VI) to U(V) (151). Because U(V) is unstable, it is then abiotically disproportionated to form U(IV) and U(VI) (151).

2.6.3 Products of U(VI) Reduction

Until 2005, the only reported product of U(VI) reduction was UO_2. UO_2 is an insoluble mineral that consists of a lattice of U(IV) and oxygen atoms (Figure 2.6) (152). Recently, studies have demonstrated that biogenic U(VI) reduction forms nanoparticulate UO_2 that is typically only 2 to 3 nm in diameter (125, 151-155). Whereas nanoparticulate biogenic UO_2 has been shown to be as stable as larger, synthetic UO_2 under anoxic conditions and at neutral pH (156), both biogenic and synthetic UO_2 are oxidized and re-solubilized by oxygen exposure (153, 157). Therefore, other forms of insoluble reduced U(IV) are of interest.

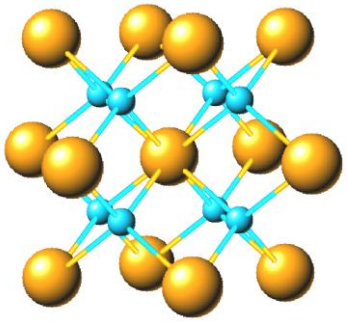

Figure 2.6 The structure of UO_2, including near-neighbor oxygen (blue) and uranium (yellow) shells surrounding the central U atom (adapted from (158)).

Three studies have reported the production of a U(IV) product other than UO_2 (Table 2.5): Khijniak et al. reported the production of ningyoite [$CaU(PO_4)_2 \times H_2O$] from uramphite [$(NH_4)(UO_2)(PO_4) \times 3H_2O$] by *Thermoterrabacterium ferrireducens*, Francis and Dodge reported the production of U(IV)-citric acid from U(VI)-citric acid by *Clostridium* spp. (159), and Junier et al. reported the production of an unidentified, non-UO_2 solid by *Desulfotomaculum reducens* strain MI-1 (160). Most U(VI) reduction assays supply U(VI) as either uranyl acetate or uranyl carbonate (Table 2.5) (125, 146-148, 153-154, 161-162); therefore, the production of uramphite and U(IV)-citric acid was likely impacted by the initial form of U(VI) provided. The formation of reduced products other than UO_2 is likely controlled by both biotic factors (e.g., organism-specific traits such as extracellular features) and abiotic factors (e.g., the initial form of U(VI) provided, the solution composition). Identifying and characterizing unique U(VI) reduction products is critical for predicting the mobility and fate of uranium in situ

Table 2.4 Identified products of U(VI) reduction.

Organism	Gram Stain	U(VI) Provided	U(IV) Produced	Resting Cells	Assay Conditions	Reference
Anaeromyxobacter dehalogenans 2CP-C	Negative	U(VI) carbonate	UO_2	yes	Bicarbonate buffer	(146)
Anaeromyxobacter dehalogenans	Negative	U(VI) acetate	UO_2	yes	Bicarbonate and PIPES[a] buffer	(147)
Clostridium spp.	Positive	U(VI)-citric acid	U(IV)-citric acid	yes	Mineral salts medium	(159)
Desulfosporosinus P3	Positive	U(VI) acetate	UO_2	yes	Unbuffered water	(131)
Desulfosporosinus spp.	Positive	U(VI) contamination	UO_2	no	Groundwater and sediments	(154)
Desulfotomaculum reducens MI-1	Positive	NR[b]	Not UO_2	no	NR	(160)
Desulfovibrio desulfuricans	Negative	U(VI) carbonate	UO_2	yes	Bicarbonate buffer	(145)
Desulfovibrio vulgaris	Negative	U(VI) acetate	UO_2	yes	Bicarbonate and PIPES buffer	(147)
Geobacter metallireducens	Negative	U(VI) carbonate	UO_2	yes	Bicarbonate buffered groundwater	(157)
Geobacter sulfurreducens	Negative	U(VI) carbonate	UO_2	yes	Bicarbonate buffer	(151)
Geobacter sulfurreducens	Negative	U(VI) acetate	UO_2	yes	Bicarbonate and PIPES buffer	(147)
Shewanella oneidensis MR-1	Negative	U(VI) carbonate	UO_2	yes	Bicarbonate buffer	(148)
Shewanella oneidensis MR-1	Negative	U(VI) acetate	UO_2	yes	Bicarbonate buffer	(153)
Shewanella oneidensis MR-1	Negative	U(VI) acetate	UO_2	yes	PIPES buffered artificial groundwater	(153)
Shewanella oneidensis MR-1	Negative	U(VI) acetate	UO_2	yes	Bicarbonate and PIPES buffer	(161)
Shewanella oneidensis MR-1	Negative	U(VI) acetate	UO_2	yes	Bicarbonate and PIPES buffer	(147)
Shewanella putrefaciens CN32	Negative	U(VI) carbonate	UO_2	yes	Bicarbonate buffer	(144)
Shewanella putrefaciens CN32	Negative	U(VI) carbonate	UO_2	yes	Bicarbonate buffer	(125)
Shewanella putrefaciens CN32	Negative	U(VI) acetate	UO_2	yes	Bicarbonate and PIPES buffer	(147)
Shewanella sp. HRCR-2	Negative	U(VI) acetate	UO_2	yes	Bicarbonate and PIPES buffer	(147)
Thermoterrabacterium ferrireducens	Positive	U(VI) phosphate	Ningyoite	no	Bicarbonate buffer	(134)

[a] PIPES is piperazine-N,N′-bis(2-ethanesulfonic acid).
[b] NR indicates that either the type of U(VI) provided or the assay conditions were not reported.

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CHAPTER 3

ELECTRON DONOR AVAILABILITY FOR MICROBIAL REDUCTIVE PROCESSES FOLLOWING THERMAL TREATMENT

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3.1 Abstract

Coupling thermal treatment with bioremediation promises effective remediation of chlorinated solvent contaminated sites. Thermal treatment removes significant free-phase solvent mass and may also enhance the effectiveness of bioremediation by establishing temperature gradients and increasing electron donor availability. Microcosms were constructed with tetrachloroethene- (PCE) and trichloroethene- (TCE) impacted soils from Great Lakes, IL and Ft. Lewis, WA and were incubated at temperatures between 24 and 95°C for four months. Incubation at elevated temperatures promoted the release of matrix-bound organic carbon but decreased general metabolic activity and limited dechlorination by indigenous microbial communities. Five days after microcosms were cooled to 24°C and bioaugmented with the methanogenic, PCE-to-ethene dechlorinating OW consortium, at least 85% of PCE and TCE were dechlorinated, but dechlorination ceased prior to complete conversion to ethene. Biostimulation with hydrogen gas circumvented the dechlorination stall and complete dechlorination to ethene

occurred in most microcosms. The majority of the electron donor liberated during heating was consumed in methanogenesis rather than reductive dechlorination, but CH_4 concentrations were also significantly positively correlated ($p < 0.05$) with previous incubation temperature.

3.2 Introduction

Chlorinated ethenes are toxins that are widespread in groundwater and are often present in the subsurface as dense non-aqueous phase liquids (DNAPLs) (1). DNAPLs may serve as long-term sources of contamination (1). In situ thermal treatment coupled with soil vapor extraction can remove a significant fraction of chlorinated solvent DNAPL mass (2); however, like other source zone remedies, thermal treatment does not remove 100% of the DNAPL mass (3-5). Anaerobic bioremediation has been employed successfully for remediation of chlorinated ethene plumes (6) and recent studies have suggested that bioremediation may be an effective polishing step following thermal treatment (4, 7). Anaerobic bioremediation of PCE and TCE occurs via stepwise reductive dechlorination reactions, i.e., PCE is transformed to TCE, TCE is transformed to dichloroethene (DCE), DCE is transformed to vinyl chloride (VC), and VC is transformed to non-toxic ethene. Although numerous microbes dechlorinate PCE and TCE to *cis*-DCE, strictly hydrogenotrophic *Dehalococcoides* spp. (*Dhc*) are the only known organisms capable of transforming DCEs to ethene (8).

Dhc require appropriate subsurface environments (i.e., temperature, redox conditions, pH) and hydrogen as electron donor (6). A previous study characterized the redox conditions and pH in sediments collected prior to electrical resistance heating

(ERH) and approximately 6 months after the completion of ERH. The pH was 0.5 units lower in the heated sediments, but both heated and unheated sediments were anaerobic (9). Therefore, thermal treatment may have little impact on pH and redox conditions. Conversely, thermal treatment obviously affects subsurface temperatures as source zones are routinely heated to temperatures of 100°C or greater (2).

Optimal temperatures for complete microbial reductive dechlorination to ethene are 25-30°C, higher than those of most aquifers, but much lower than source zone temperatures during thermal treatment (10-11). Therefore, dechlorination activity is unlikely to occur in the source zone during treatment and, it is also unlikely that native *Dhc* will remain viable (12). Because a prerequisite for successful bioremediation is the presence of viable *Dhc*, following thermal treatment, a *Dhc*-containing dechlorinating consortium will likely have to be amended to the subsurface via bioaugmentation (6, 13-14). Interestingly, because of the temperature gradient developed during thermal treatment, dechlorination may actually be enhanced in the perimeter of the source zone.

Previous studies have reported that organic carbon is released from the subsurface matrix during thermal treatment, increasing electron donor availability for dechlorinating organisms (7, 15). The released organic carbon, suggested to be in the form of long-chain fatty acids (7), cannot directly serve as an electron donor for *Dhc* and must be fermented to produce hydrogen. Furthermore, *Dhc* must compete with other organisms, such as methanogens, for electron donor. In a previous study, less methanogenesis was observed in previously heated microcosms than in those that were never heated, suggesting that competitors to dechlorinators are less successful following thermal treatment (15). Therefore, thermal treatment may both increase electron donor

availability and decrease the activity of competitors to dechlorinators. If this is the case, dechlorination by bacteria bioaugmented to the source zone may be entirely fueled by the available electron equivalents and biostimulation, (the introduction of additional electron donor), may not be required.

This study focuses on (i) the potential for dechlorination activity in the perimeter of the source zone during thermal treatment and (ii) the impact of competitors (e.g., methanogens) to dechlorinators on electron donor availability. Microcosms were constructed with soils from two chlorinated ethene-contaminated sites, one with high levels of PCE contamination and one with low levels of TCE contamination. After four months of incubation at 24, 35, 50, 70, and 95ºC, microcosms were cooled to 24ºC, and bioaugmented with a methanogenic dechlorinating consortium. Throughout the experiment, reductive dechlorination and methanogenesis were monitored to determine whether the success of dechlorinators and/or their competitors correlated with previous incubation temperature.

3.3 Materials and Methods

3.3.1 Site Description and Soil Preparation

Soil and groundwater samples impacted with trace concentrations (less than 1 mg/L) of TCE and *cis*-DCE were collected from the East Gate Disposal Yard (EGDY) in Fort Lewis, WA from 28 to 36 feet below ground surface (see Friis et al., 2007 for a description of the Ft. Lewis site). Soil and groundwater samples impacted with PCE and trace concentrations TCE, *cis*-DCE, and VC were collected from a former dry cleaner facility at the Naval Training Center in Great Lakes, IL from between 8 to 10 feet below

ground surface. A slurry of Great Lakes soil and groundwater was prepared at room temperature (24ºC) in a disposable glove bag filled with ultra high purity argon. Soil and groundwater from the Great Lakes site were combined in equal masses and the soil was broken into fragments with diameters no larger than 10 mm.

3.3.2 Microcosm Construction, Incubation Conditions, and Sampling

A total of 30 Ft. Lewis microcosms were prepared at room temperature in a glove box (Coy Laboratory Products, Ann Arbor, Michigan) that contained a 95% N_2 / 5% H_2 atmosphere. Serum bottles (70 mL) were filled with 15 mL groundwater and soil was added to a total volume of approximately 37 mL. A total of 20 Great Lakes microcosms were constructed at room temperature in an argon-filled glove bag. In 70-mL serum bottles, the soil and groundwater slurry was combined with 10 to 20 mL of sterile mineral salts medium or site groundwater to a total volume of 40 mL. All microcosms were capped with sterile black butyl-rubber stoppers prior to removal from the glove box or argon-filled bag. Mineral salts medium was prepared as described (16), except that N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid was omitted and the medium contained 0.2 mM Na_2S x 9 H_2O (Sigma-Aldrich Co., St. Louis, MO). Immediately following microcosm preparation, five Great Lakes and fifteen Ft. Lewis microcosms were autoclaved at 121^{o}C for 30 minutes to serve as autoclaved controls. Triplicate Great Lakes and Ft. Lewis unautoclaved microcosms and a single Great Lakes and triplicate Ft. Lewis autoclaved controls were incubated at 24, 35, 50, 70, and 95ºC. The temperature in microcosms incubated at 35 and 50ºC was increased by 1ºC per day until reaching the target temperature. All other microcosms were placed at the target temperature

immediately after preparation. After 27 days of incubation, Ft. Lewis microcosms were amended with 1 µL of TCE dissolved in 2.25 mL filter sterilized, anoxic groundwater from the Ft. Lewis site. Following 4 months of incubation, microcosm temperatures were decreased by 5ºC per day to 24ºC. Microcosms were sampled periodically for chlorinated ethenes and gases, including acetylene, CH_4, CO_2, and hydrogen. Aqueous and gaseous samples were removed from microcosms using sterile syringes and needles. Removed aqueous volumes were replaced with filter-sterilized groundwater or sterile medium and removed gaseous volumes were replaced with sterile N_2 gas.

3.3.3 Enrichment Culture Preparation, Incubation Conditions, Sampling, and Transfers

Prior to bioaugmentation, but after 138 days of incubation, enrichment cultures were constructed from unautoclaved Ft. Lewis microcosms previously incubated at 35°C. From each of the three microcosms, 2 mL aqueous volumes were removed and used as the inocula to three individual 160 mL serum bottles. Serum bottles contained approximately 100 mL mineral salts medium amended with vitamins (17), 5 mM lacate as electron donor, and 0.9 µL TCE. Enrichment cultures were initially incubated at 24°C and aqueous samples were removed periodically for chlorinated ethene quantification. Once TCE was completely dechlorinated to *cis*-DCE during incubation at 24°C, enrichment cultures were transferred to 35°C and amended with 0.9 µL TCE. To ensure that dechlorination activity was reproducible, once dechlorination to *cis*-DCE occurred during incubation at 35°C, 3 mL aqueous volumes from enrichment cultures were

transferred to fresh bottles containing amended mineral salts medium and cultures were again incubated at 35°C.

3.3.4 Bioaugmentation and Biostimulation

After microcosms were cooled to 24ºC, 10 mL of OW, a mixed, methanogenic, PCE-to-ethene-dechlorinating consortium was added to all microcosms. Culture OW contains multiple *Dehalococcoides* strains along with *Geobacter*, *Dehalobacter*, and *Sulfurospirillum* populations likely capable of PCE-to-*cis*-DCE dechlorination (18). Prior to inoculation, medium containing the OW consortium was removed from a draw-and-fill bioreactor (18) and sparged with N_2 gas for 30 minutes. Therefore, whereas the inoculum likely contained acetate and propionate (18), only trace concentrations of chlorinated ethenes, ethene, and CH_4 were amended to microcosms.

All microcosms were biostimulated with hydrogen gas. The initial biostimulation event in all microcosms and the second biostimulation event in Ft. Lewis microcosms were performed by amending 5 mL of sterile hydrogen gas via syringe to each microcosm. Secondary biostimulation in Great Lakes microcosms was performed by repeatedly amending microcosms with 5 mL of sterile hydrogen gas two times weekly.

3.3.5 Analytical Methods and Calculations

Aqueous samples (1 mL) were collected for the quantification of chlorinated ethene and ethene concentrations by gas chromatography (GC) (16). Gaseous samples (2 mL) were collected for the quantification of acetylene, CH_4, CO_2, and hydrogen using a

Hewlett Packard 6890 GC equipped with a heated gas sampling valve, a 250 µL sample loop, and a 30 m by 0.32 mm OD Carboxen-1010 column (Supelco, Bellefonte, PA) connected to a thermal conductivity detector.

Concentrations of chlorinated ethenes, ethene, and CH_4 were converted to molar values by accounting for partitioning between the aqueous and vapor phase using published Henry's constants and assuming that no sorption occurred (19). The moles of electron equivalents used for methanogenesis was calculated by assuming that four moles of electron equivalents are required per mole of CH_4 formed (20). To calculate the moles of electron equivalents consumed for reductive dechlorination, it was assumed that each dechlorination step required two electrons (21) and therefore, the moles consumed were calculated for the Great Lakes microcosms according to the formula:

$$2(M_{TCE}) + 4(M_{DCEs}) + 6(M_{VC}) + 8(M_{ethene}) = M_{consumed} \quad (3.1)$$

where M_{TCE} is the number of moles of TCE, M_{DCEs} is the sum of the moles of DCEs, M_{vc} is the moles of vinyl chloride, M_{ethene} is the moles of ethene, and $M_{consumed}$ is the number of moles of electron equivalents consumed for reductive dechlorination. The moles of electron equivalents used for reductive dechlorination in Ft. Lewis microcosms was calculated according to the formula:

$$2(M_{DCEs}) + 4(M_{VC}) + 6(M_{ethene}) = M_{consumed} \quad (3.2)$$

Dechlorination extent in Great Lakes microcosms was determined according to the formula (9):

$$[1(M_{TCE}) + 2(M_{DCE}) + 3(M_{VC}) + 4(M_{ethene})]/[4(M_{tot})] = DE \quad (3.3)$$

where M_{DCE} is the moles of *cis*-DCE, M_{tot} is the total number of moles of chlorinated ethenes and ethene, and DE is the dechlorination extent. In Ft. Lewis microcosms, dechlorination extent was determined according to the formula:

$$[1(M_{DCE}) + 2(M_{VC}) + 3(M_{ethene})]/[3(M_{tot})] = DE \quad (3.4)$$

Concentrations in microcosms were compared using two-tailed student's t-tests and correlations were described based on Pearson product moment correlation coefficients. In all cases, p-values below 0.05 were considered significant. Principal component analysis (PCA) was performed with the ViSta program (22).

3.4 Results

3.4.1 Incubation at Elevated Temperatures

After 28 days of incubation, hydrogen concentrations in unautoclaved Ft. Lewis microcosms incubated at 24, 35, and 50ºC were below the detection limit (40 ppmv), 600 ± 1040, and 1090 ± 1888 ppmv, respectively. These concentrations were significantly lower ($p < 0.05$) than the concentrations in autoclaved microcosms incubated at the same temperatures, (16,460 ± 2,900, 19,260 ± 4,010, and 18,280 ± 3,020 ppmv, respectively). For the duration of the 120-day incubation period, hydrogen concentrations were significantly lower ($p < 0.05$) in unautoclaved microcosms incubated at 24, 35, and 50°C than in autoclaved microcosms incubated at the same temperatures. Conversely, after 28 days of incubation, hydrogen concentrations in unautoclaved microcosms incubated at 70 and 95°C (19,850 ± 630 and 16,890 ± 230 ppmv, respectively) were not significantly lower than concentrations in autoclaved microcosms (19,760 ± 1,200 and 15,670 ± 810

ppmv, respectively) and throughout the 120-day incubation period, hydrogen concentrations were never significantly lower in unautoclaved microcosms incubated at 70 and 95ºC than in autoclaved microcosms incubated at the same temperatures.

After 28 days of incubation, CO_2 concentrations in all unautoclaved Ft. Lewis microcosms except those previously incubated at 95°C were significantly lower ($p < 0.05$) than concentrations in autoclaved microcosms incubated at the same temperatures. CO_2 concentrations in unautoclaved microcosms after 28 days of incubation ranged from 1,830 ± 930 to 10,360 ± 245 ppmv in microcosms incubated at 24 and 95°C, respectively, and were positively correlated with incubation temperature ($p < 0.05$). Over the course of the 120 day incubation period, CO_2 concentrations increased in all unautoclaved microcosms and CO_2 concentration was positively correlated with incubation time ($p < 0.05$) in unautoclaved microcosms incubated at 24, 35, 50, and 70°C. Conversely, CO_2 concentration and incubation time were not positively correlated in autoclaved microcosms. In unautoclaved microcosms, CO_2 production rates increased exponentially with decreasing temperature (Figure 3.1). Based on the Arrhenius equation, the apparent activation energy for methane production was 23.6 kJ/mol.

After 120 days of incubation, TCE persisted in all Ft. Lewis microcosms incubated at 35, 50, 70, and 95°C and in autoclaved microcosms incubated at 24°C. TCE concentrations in all unautoclaved microcosms except those incubated at 24°C were not significantly lower than concentrations in autoclaved microcosms incubated at the same temperatures (data not shown). In unautoclaved Ft. Lewis microcosms incubated at 24ºC, TCE decreased to below the detection limit (0.05 µmole/microcosm) after 120 days of

incubation and *cis*-DCE concentrations increased concomitantly. VC and ethene were not produced in any microcosm.

In Great Lakes microcosms, hydrogen was below the detection limit in all microcosms after 58 days of incubation. After 28 days of incubation, CO_2 concentrations were lower in all triplicate unautoclaved microcosms incubated at 24, 35, 50, and 70°C (37,330 ± 2,730, 44,560 ± 6,870, 44,830 ± 4,320, and 37,620 ± 1,080 ppmv, respectively) than in autoclaved microcosms incubated at the same temperatures (53,450, 73,450, 57,970, and 48,680 ppmv, respectively). CO_2 concentrations in unautoclaved microcosms incubated at 95°C (48,670 ± 3,360 ppmv) were similar to those in the autoclaved microcosm incubated at 95°C (49,500 ppmv). In unautoclaved microcosms incubated at 24, 50, and 70°C, CO_2 concentrations were significantly positively correlated ($p < 0.05$) with incubation time and increased to 60,680 ± 5810, 64,020 ± 9,480, and 75,060 ± 1,740 ppmv, respectively, after 123 days of incubation. However, CO_2 concentrations were also significantly positively correlated ($p < 0.05$) with incubation time in autoclaved microcosms incubated at 50 and 70°C and increased to 97,460 and 70,990 ppmv, respectively, after 123 days of incubation.

After 123 days of incubation, PCE persisted in all Great Lakes microcosms. PCE concentrations in all triplicate unautoclaved microcosms incubated at 24, 35, 50, and 70°C, (31.3 ± 3.1, 22.2 ± 0.3, 16.5 ± 0.9, and 18.3 ± 1.1 µmole/bottle, respectively) were greater than those in autoclaved microcosms (17.1, 12.3, 15.4, and 16.9 µmole/microcosm, respectively). The PCE concentration in the autoclaved microcosm incubated at 95°C was within the range of concentrations measured in the unautoclaved microcosms incubated at 95°C (11.0 and 11.4 ± 1.2 µmole/microcosm, respectively).

TCE, *cis*-DCE, and VC concentrations were similar in unautoclaved and autoclaved microcosms and concentrations of PCE dechlorination products in all microcosms remained below 0.5 µmole/microcosm.

3.4.2 Cooling from Elevated Temperatures

Microcosms were cooled from elevated temperatures both in preparation for bioaugmentation and to determine whether microbial activity would resume during cooling. The concentration of hydrogen decreased significantly ($p < 0.05$), from 22,420 ± 1,170 to 18,970 ± 4,590 ppmv, during cooling in unautoclaved Ft. Lewis microcosms previously incubated at 70°C, but hydrogen concentrations did not change significantly in autoclaved microcosms previously incubated at the same temperature. Following cooling, TCE and PCE persisted in all Ft. Lewis and Great Lakes microcosms, respectively, (with the exception of unautoclaved Ft. Lewis microcosms previously incubated at 24°C). Reductive dechlorination products were not produced in any microcosm during cooling. Following cooling, PCE concentrations in Great Lakes microcosms were negatively correlated with previous incubation temperature ($p < 0.05$). Similarly, excluding unautoclaved Ft. Lewis microcosms previously incubated at 24°C, TCE concentrations in Ft. Lewis microcosms were negatively correlated with previous incubation temperature ($p < 0.05$).

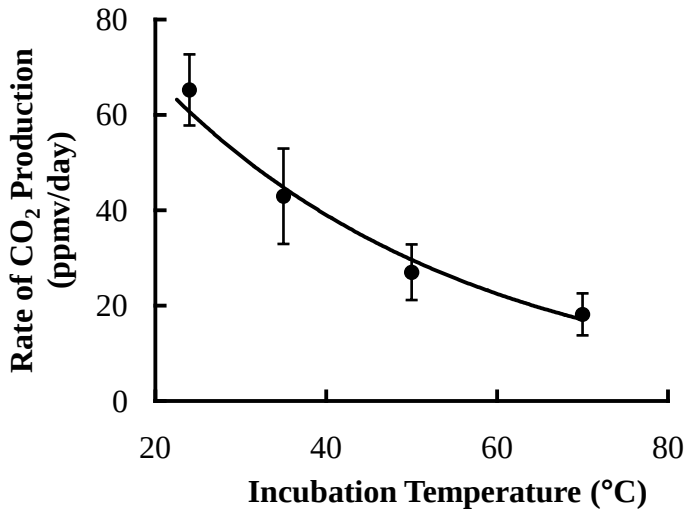

Figure 3.1 Exponential regression analysis of the rate of CO_2 production in unautoclaved Ft. Lewis microcosms incubated at temperatures of 24, 35, 50, and 70°C. The regression indicates that the rate of CO_2 production is inversely exponentially correlated with incubation temperature. Error bars represent standard errors of CO_2 production rates.

3.4.3 Reductive Dechlorination in Ft. Lewis Enrichment Cultures

In order to investigate the lack of dechlorination activity in unautoclaved Ft. Lewis microcosms previously incubated at 35°C, enrichment cultures were prepared once microcosms were cooled. Stoichiometric reductive dechlorination of TCE to *cis*-DCE occurred in 20 days during incubation at 24°C in enrichment cultures inoculated with aqueous samples from the microcosms previously incubated at 35°C. After cultures were placed at 35°C and again amended with TCE, complete dechlorination of TCE to *cis*-DCE occurred in 2 days. When culture fluid was transferred to fresh medium, TCE to *cis*-DCE reductive dechlorination activity occurred during incubation at 35°C.

3.4.4 Reductive Dechlorination Following Bioaugmentation

Because complete dechlorination of chlorinated ethenes to ethene did not occur in any microcosm during incubation at temperatures ranging from 24 to 95°C, all microcosms were bioaugmented. In Ft. Lewis microcosms, at least 95% of TCE was dechlorinated to VC and ethene within 5 days of bioaugmentation (Figure 3.2). In Great Lakes microcosms, at least 85% of PCE was dechlorinated to *cis*-DCE and VC only 3 days after bioaugmentation.

The only Ft. Lewis microcosms demonstrating a significant increase ($p < 0.05$) in the molar percentage of ethene from 10 to 13 days following bioaugmentation were those unautclaved microcosms previuosly incubated at 24ºC. No significant increase in the molar percentage of VC occurred in any of the unautoclaved Great Lakes microcosms from 11 to 23 days following bioaugmentation. In autoclaved Great Lakes microcosms, the molar percentage of VC increased by a maximum of 7.9% from 11 to 23 days of

incubation (Figure 3.2). In all Ft. Lewis and Great Lakes microcosms 13 and 23 days after bioaugmentation, respectively, hydrogen concentrations were below the detection limit. In both Ft. Lewis and Great Lakes microcosms, the moles of electron equivalents consumed for reductive dechlorination following bioaugmentation were negatively correlated with previous incubation temperature ($p < 0.05$) (Figure 3.3). This indicates that more electrons were consumed for dechlorination in microcosms incubated at lower temperatures.

3.4.5 Reductive Dechlorination Following Biostimulation

Because dechlorination ceased prior to complete conversion of chlorinated ethenes to ethene, all microcosms were biostimulated. The molar percentage of VC in Ft. Lewis microcosms decreased by 47.3 ± 34.2% 17 days after biostimulation (day 31, Figure 3.2) and, with the exception of unautoclaved microcosms previously incubated at 50 and 70°C, 90% dechlorination occurred within 42 days of biostimulation (Table 3.1). In Ft. Lewis unautoclaved microcosms previously incubated at 50 and 70°C, over 70% of chlorinated ethenes existed as VC 92 days after biostimulation in two of three and one of three microcosms, respectively (day 106, Figure 3.2). Because no hydrogen was detected in these microcosms 92 days after biostimulation, they were amended again with hydrogen. Following this second biostimulation event, the VC molar percentage decreased in all microcosms within 14 days (day 133, Figure 3.2) and 90% dechlorination occurred in 29 and 14 days in the microcosms previously incubated at 50 and 70°C, respectively (day 148 and 133, Figure 3.2).

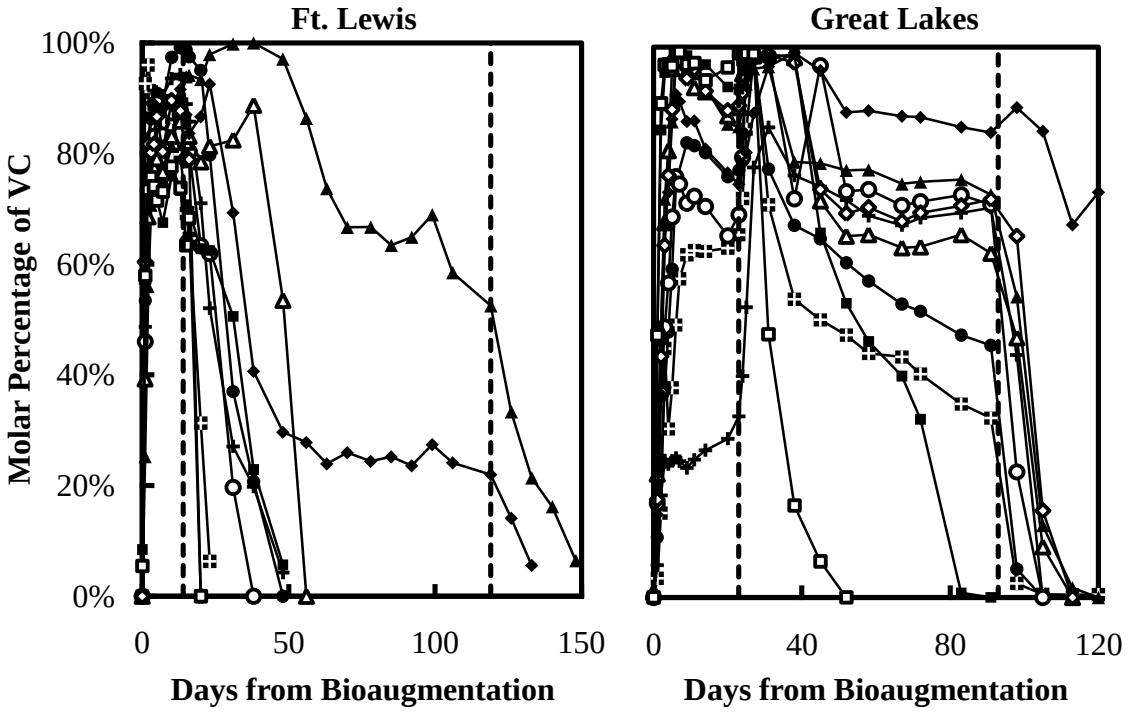

Figure 3.2 The change in the molar percentage of VC with time after bioaugmentation in Ft. Lewis and Great Lakes microcosms previously incubated at 24 (crosses), 35 (circles), 50 (triangles), 70 (diamonds), and 95°C (squares) in unautoclaved (filled symbols) and autoclaved (open symbols) microcosms. Vertical dashed lines indicate biostimulation events and symbol error bars have been omitted for clarity. The molar percentage of VC is defined as the number of moles of VC divided by the total number of moles of chlorinated ethenes and ethene.

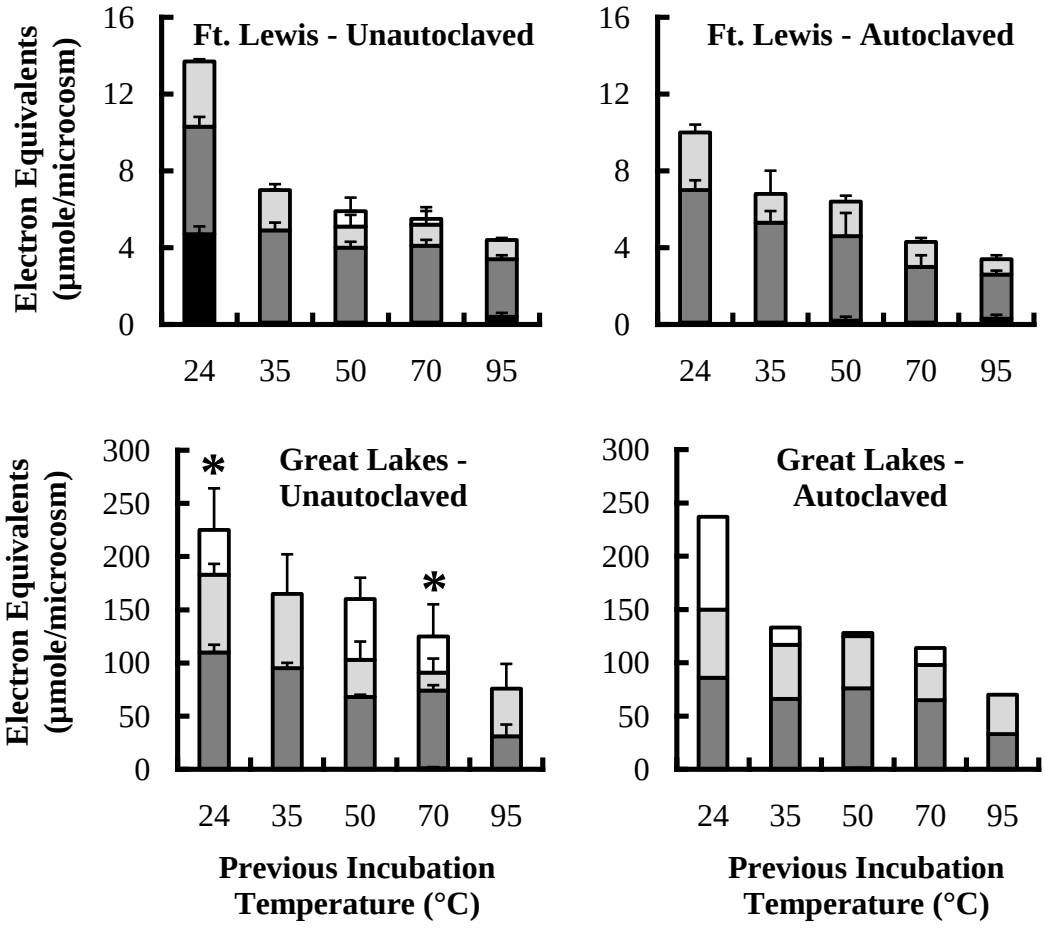

Figure 3.3 The number of electron equivalents consumed for reductive dechlorination prior to bioaugmentation (black bars), following bioaugmentation (dark gray bars), following biostimulation event 1 (light gray bars), and following secondary biostimulation (white bars). Values are shown for Ft. Lewis and Great Lakes microcosms 13 and 23 days after bioaugmentation, 119 and 91 days following bioaugmentation, and after 90% dechlorination of VC and complete dechlorination of VC, respectively. Asterisks indicate that complete dechlorination did not occur during the incubation period. Error bars represent standard error, but negative error bars have been removed for clarity. No error bars are shown for autoclaved Great Lakes microcosms because only one microcosm was incubated at each temperature.

In Great Lakes microcosms, the molar percentage of VC increased by 11.3 ± 7.4% due to the conversion of *cis*-DCE to VC 2 days after biostimulation (day 25, Figure 3.2). In autoclaved and unautoclaved Great Lakes microcosms previously incubated at 95°C, all VC was converted to ethene 29 and 60 days after biostimulation, respectively (day 52 and 83, Figure 3.2). The molar percentage of VC did not decrease significantly from 60 to 68 days following biostimulation in unautoclaved Great Lakes microcosms previously incubated at temperatures below 95°C (day 83 to 91, Figure 3.2). In autoclaved Great Lakes microcosms previously incubated at the same temperatures, the molar percentage of VC decreased by a maximum of 3% during the same period (day 83 to 91, Figure 3.2). No hydrogen was detected in any Great Lakes microcosm 68 days after biostimulation (day 91, Figure 3.2) and therefore, microcosms were subjected to secondary, repeated biostimulation. Following 12 days of secondary biostimulation, the molar percentage of VC decreased by 41.6 ± 24.1% (day 105, Figure 3.2). Within 27 days of secondary biostimulation (day 120, Figure 3.2), VC was below the detection limit (0.05 µmole/microcosm) in all microcosms except unautoclaved microcosms previously incubated at 24 and 70°C (Table 3.1). In unautoclaved microcosms previously incubated at 24 and 70°C, the VC molar percentages were 0.5 ± 0.1% and 73.6 ± 37.4% after 27 days of secondary biostimulation (day 120, Figure 3.2). The time required for 90% dechlorination in Ft. Lewis microcosms and 100% dechlorination of VC in Great Lakes microcosms was not correlated with previous incubation temperature.

Table 3.1 The total number of days following bioaugmentation required for 90% of VC to be dechlorinated to ethene and for complete dechlorination of VC to ethene in Ft. Lewis and Great Lakes microcosms, respectively. The numbers of days following biostimulation 1/biostimulation 2 are listed in parentheses. Dashes indicate that secondary biostimulation was not required and asterisks indicate that complete dechlorination did not occur during the incubation period.

Previous Incubation Temperature	Days for Dechlorination to Ethene			
	Ft. Lewis Microcosms		Great Lakes Microcosms	
	Unautoclaved	Autoclaved	Unautoclaved	Autoclaved
24°C	23 (9/-)	48 (34/-)	141* (118/48)	105 (82/12)
35°C	48 (34/-)	38 (24/-)	105 (82/12)	105 (82/12)
50°C	148 (134/29)	56 (42/-)	120 (97/27)	113 (90/20)
70°C	133 (119/14)	20 (6/-)	141* (118/48)	113 (90/20)
95°C	48 (34/-)	20 (6/-)	83 (60/-)	52 (29/-)

3.4.6 Competition for Electron Donors

Prior to bioaugmentation, CH_4 was below the detection limit in all microcosms (600 ppmv). Within 23 days of bioaugmentation, average CH_4 concentrations were at least 21,000 ppmv (Figure 3.4). The ratio of the number of electron equivalents consumed for methanogenesis to electron equivalents consumed for reductive dechlorination prior to biostimulation was a minimum of 1,300 indicating that, at most, 0.08% of the available reducing equivalents were consumed for reductive dechlorination. CH_4 concentrations in unautoclaved Ft. Lewis microcosms previously incubated at 24 and 35°C were significantly higher ($p < 0.05$) than concentrations in autoclaved microcosms incubated at the same temperatures, but concentrations in unautoclaved and autoclaved microcosms previously incubated at 50, 70, and 95°C were not significantly different.

Using PCA, CH_4 concentrations, the number of electrons consumed for reductive dechlorination, and the dechlorination extent in Great Lakes microcosms following bioaugmentation were visually compared (Figure 3.5). Data points representing microcosms previously incubated at lower temperatures generally have lower PC 1 values (Figure 3.5) and, in fact, previous incubation temperature is significantly positively correlated ($p < 0.05$) with CH_4 concentration. CH_4 concentrations were significantly negatively correlated ($p < 0.05$) with the moles of electrons consumed for reductive dechlorination, but were significantly positively correlated ($p < 0.05$) with dechlorination extent. That is, generally, microcosms that produced more CH_4 consumed fewer electrons for reductive dechlorination. These microcosms, however, also had

higher dechlorination extents because lower concentrations of PCE were present prior to bioaugmentation.

In Ft. Lewis microcosms, CH_4 concentrations increased by over 30,000 ppmv following the initial biostimulation and increased by an average of 13,000 ppmv following secondary biostimulation (Figure 3.4). The CH_4 concentration, time required for dechlorination, and number of electron equivalents consumed for reductive dechlorination in Ft. Lewis microcosms following 90% VC dechlorination were compared using PCA (Figure 3.5). Data points representing microcosms previously incubated at lower temperatures generally have lower PC 1 values (Figure 3.5) because previous incubation temperature is significantly positively correlated ($p < 0.05$) with CH_4 concentration and significantly negatively correlated ($p < 0.05$) with the number of electrons consumed for reductive dechlorination. These results demonstrate that more CH_4 was produced while fewer electron equivalents were required for 90% VC dechlorination in microcosms previously incubated at higher temperatures.

In all Great Lakes microcosms except for those previously incubated at 50°C, at least 19,000 ppmv CH_4 was produced following the initial biostimulation and prior to secondary, repeated biostimulation (Figure 3.4). During the period following the initial biostimulation and prior to secondary biostimulation, no CH_4 was produced in the unautoclaved microcosms previously incubated at 50ºC and CH_4 concentrations increased by less than 3,000 ppmv in autoclaved microcosms previously incubated at 50°C (Figure 3.4). During secondary biostimulation, CH_4 concentrations increased by at least 74,000 ppmv and increases in concentration were not correlated with previous incubation temperature. Final CH_4 concentrations in Great Lakes microcosms were not correlated

with previous incubation temperature or the number of electron equivalents consumed for dechlorination.

3.5 Discussion

3.5.1 Metabolic Activity and the Release of Soil-Bound Carbon during Incubation at Elevated Temperatures

Metabolic activity occurred in Ft. Lewis microcosms incubated at lower temperatures as evidenced by the consumption of hydrogen and the production of CO_2, a result which is in agreement with previous studies conducted with soils from the same site (7, 10). Little metabolic activity was apparent in Great Lakes microcosms. Although CO_2 concentrations were significantly positively correlated ($p < 0.05$) with incubation time in unautoclaved Great Lakes microcosms incubated at 24, 70, and 95°C, this was also true of the autoclaved microcosms incubated at 70 and 95°C, suggesting that the increase in CO_2 was due to abiotic CO_2 production during incubation at higher temperatures. Interestingly, in both Ft. Lewis and Great Lakes microcosms, exposure to elevated temperatures, including during the autoclaving process, caused either the abiotic production of CO_2. Whereas previous studies have demonstrated that sediment-bound organic carbon is released during the heating of soils (7, 15, 23-24), the increases in CO_2 concentrations measured in these microcosms may also be due to carbonate dissolution. Carbonate dissolution may play a particularly important role in the increases in CO_2 concentrations in the Great Lakes microcosms that contained bicarbonate-buffered medium. Even so, the results from the current study suggest that organic carbon may also be released as CO_2 during the heating of soils. Because many methanogens are

capable of using CO_2 as an electron acceptor (20) and compete with dechlorinators for electron donor(s) (25), the release of CO_2 may increase competition for electron donor.

3.5.2 Chlorinated Ethene Concentrations Following Cooling

Differences in the concentrations of PCE and TCE between microcosms after cooling could be accounted for by biotic reductive dechlorination, abiotic degradation, or loss of TCE and PCE from the microcosms. Because concentrations in autoclaved microcosms were either similar to or less than concentrations in unautoclaved microcosms (with the exception of the Ft. Lewis microcosms incubated at 24°C), it is unlikely that biotic dechlorination occurred in the Ft. Lewis microcosms incubated at 35, 50, 70, and 95°C or in any of the Great Lakes microcosms. Previous studies conducted with soils collected from the same sites demonstrated that the half-life of PCE is ca. 7,000 days during incubation at 95°C (26) and that the half-life of TCE is ca. 640 days during incubation at 95°C (10). Therefore, the differences in PCE and TCE concentrations are likely primarily due to diffusion through or sorption to the rubber stopper rather than due to biotic or abiotic degradation. This conclusion is consistent with a previous report that demonstrated that the loss of PCE and TCE from vials sealed with polymer septa and incubated at 50°C was due to diffusion through and sorption to the septa (27). Therefore, in the case of the Ft. Lewis and Great Lakes sites, little abiotic or biotic degradation of PCE and TCE is likely to occur in the heated source zone during or immediately following thermal treatment.

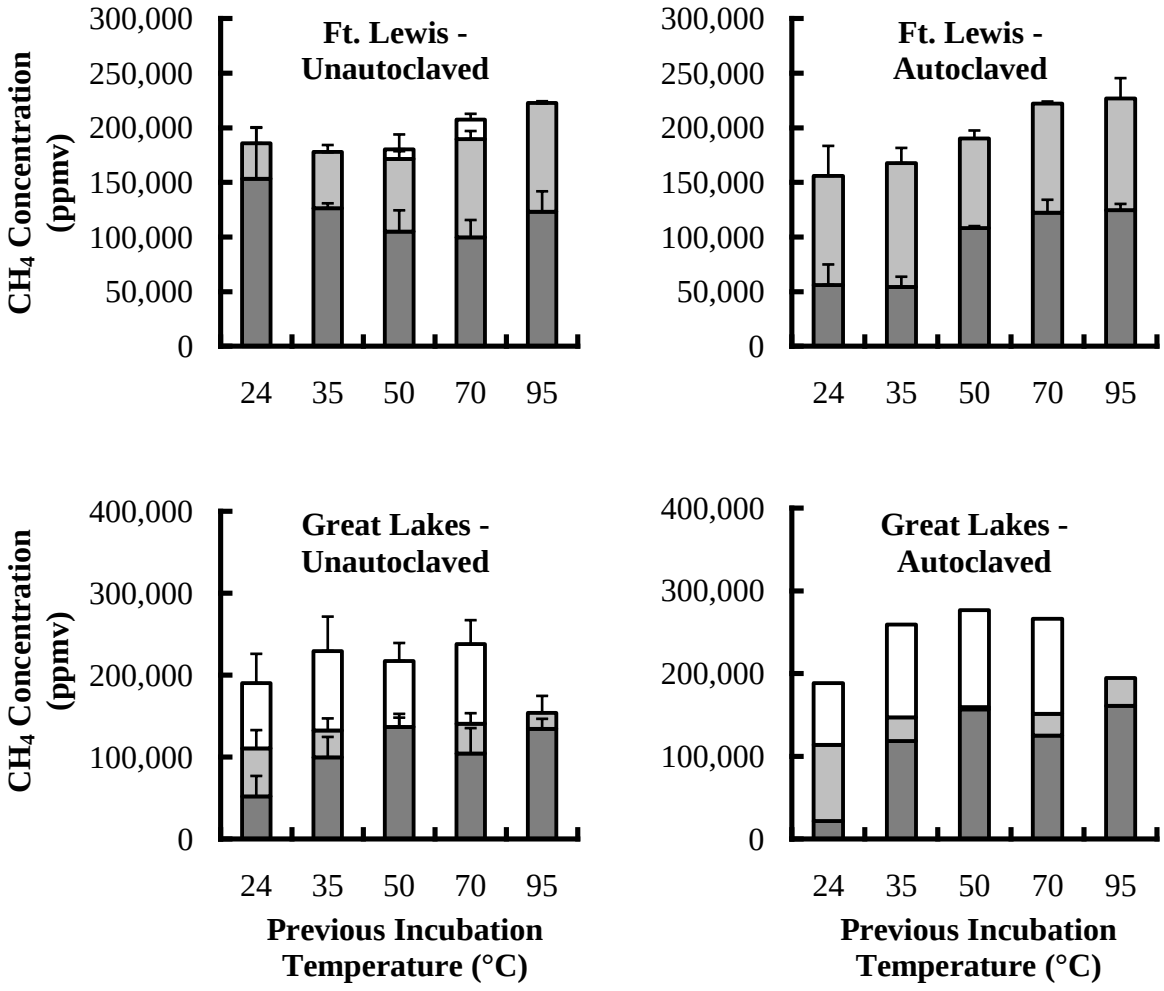

Figure 3.4 The concentration of CH_4 prior to biostimulation (dark gray bars), following initial biostimulation (light gray bars), and following secondary biostimulation (white bars). Error bars represent standard error, but negative error bars have been removed for clarity. No error bars are shown for autoclaved Great Lakes microcosms because only one microcosm was incubated at each temperature.

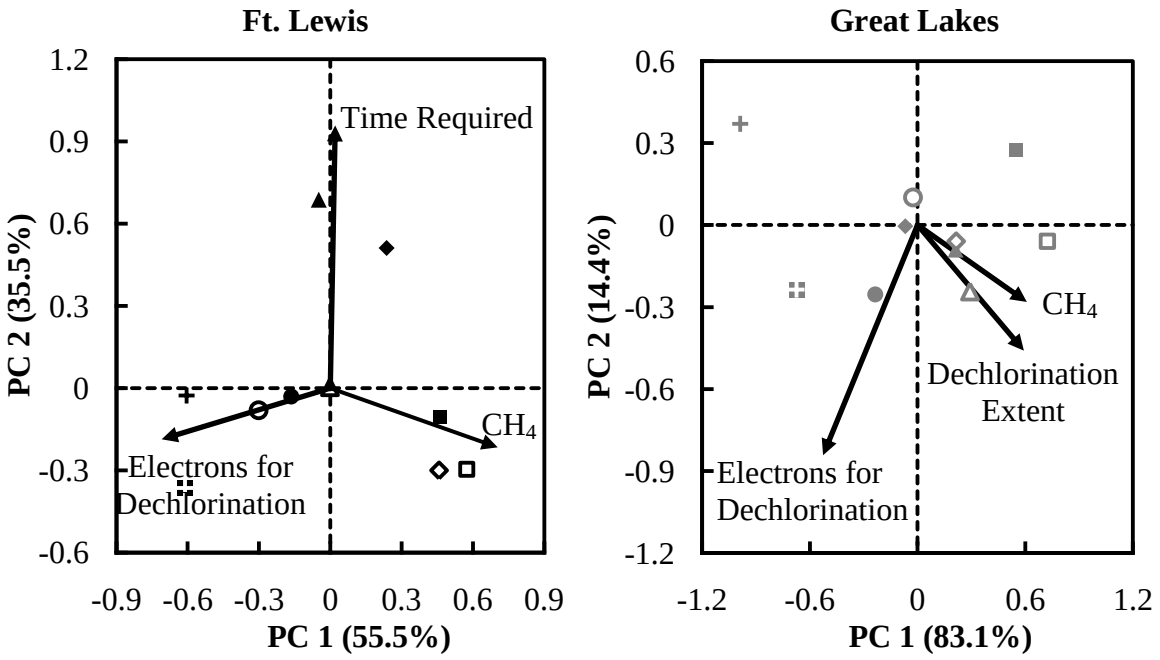

Figure 3.5 PCA ordination plots of Ft. Lewis microcosms after 90% VC dechlorination and of Great Lakes microcosms prior to biostimulation. Vectors represent CH_4 concentration, electrons consumed for dechlorination, time required for 90% VC dechlorination (Ft. Lewis plot only), and dechlorination extent (Great Lakes plot only) and symbols represent microcosms incubated at 24 (crosses), 35 (circles), 50 (triangles), 70 (diamonds), and 95°C (squares) in unautoclaved (filled symbols) and autoclaved (unfilled symbols) Ft. Lewis (black symbols) and Great Lakes (gray symbols) microcosms. The percentage of the total variation explained by each PC is shown in parentheses.

3.5.3 Reductive Dechlorination Activity and Potential Prior to Bioaugmentation

In previous studies with soils collected from Ft. Lewis, reductive dechlorination of TCE to *cis*-DCE occurred in unamended microcosms incubated at 10 and 25°C (10-11). Similarly, in the current study, prior to bioaugmentation, reductive dechlorination occurred in Ft. Lewis microcosms incubated at 24°C. Dechlorination did not occur in microcosms incubated at higher temperatures even though enrichment culture experiments demonstrated that organisms capable of dechlorination of TCE to *cis*-DCE at 35°C were present. Because increased temperatures likely affect the activity of fermenters that generate electron donors for dechlorinators as well as the activity of competitors to dechlorinators, electron donor availability may have limited reductive dechlorination in microcosms incubated at 35°C (28-30). The results of the current study demonstrate that even temperatures that are not inhibitory to dechlorinators can limit dechlorination activity via changing electron donor availability. Therefore, without electron donor amendment, dechlorination may not occur at the intermediate temperatures present in the perimeter of the source zone even when native bacteria capable of dechlorination are present. A previous study suggested that organic carbon released in the treatment zone is transported downstream with groundwater flow (9). During field-scale application of thermal treatment, this transport of organic carbon away from the treatment zone may increase electron donor availability for dechlorination at the intermediate temperatures present in the perimeter of the source zone.

3.5.4 Reductive Dechlorination Following Bioaugmentation

Complete dechlorination of PCE and TCE to ethene did not occur in any of the Great Lakes and Ft. Lewis microcosms and, therefore, all microcosms were bioaugmented. Immediately following bioaugmentation, dechlorination activity was rapid; however, following 13 and 23 days of incubation in Ft. Lewis and Great Lakes microcosms, respectively, dechlorination activity ceased. Because hydrogen concentrations were below the detection limit in all microcosms during the cease in activity, the accumulation of *cis*-DCE and VC and the stall in reductive dechlorination activity was likely due to electron donor limitations. Previous studies have proposed that heating soils increases bioavailable organic carbon concentrations, thus providing substrate(s) for hydrogen-releasing fermentation reactions (7, 15, 23-24). The results of the current study demonstrate that insufficient electron donor was present to support complete and rapid dechlorination of *cis*-DCE to VC and VC to ethene even following the heating of soils. In fact, the number of electrons consumed for reductive dechlorination prior to the stall in dechlorination was signficantly negatively correlated with previous incubation temperature; therefore, either less electron donor was available in the microcosms previously incubated at elevated temperatures or the available electron donor was consumed by competitors to dechlorinating organisms. On average, an order-of-magnitude more electrons were consumed for reductive dechlorination in Great Lakes microcosms than in Ft. Lewis microcosms prior to the stall in dechlorination activity. Interestingly, the major product in almost all Ft. Lewis and Great Lakes microcosms was VC, suggesting that VC-dechlorianting organisms could not compete successfully and that VC accumulation may occur *in situ* when inadequate electron donor is present.

3.5.5 Reductive Dechlorination Following Biostimulation

In order to achieve rapid and complete reductive dechlorination to ethene, microcosms were biostimulated. Following biostimulation, dechlorination activity resumed in most Ft. Lewis and Great Lakes microcosms within 1 week, supporting the conclusion that the stall in dechlorination activity was likely due to electron donor limitations. In Ft. Lewis unautoclaved microcosms previously incubated at 50 and 70°C, the first biostimulation event did not stimulate complete VC dechlorination. The need for additional biostimulation in certain microcosms could be due to i) the presence of higher concentrations of chlorinated solvents or more chlorinated compounds (i.e., the presence of *cis*-DCE rather than VC), ii) increased competition for electron donor, or iii) conditions inhibitory to dechlorinators. In the case of Ft. Lewis microcosms, more electrons were consumed for reductive dechlorination in almost all of the microcosms in which 90% VC dechlorination occurred than in the microcosms in which it did not. This demonstrates that the second biostimulation event was required due to the consumption of less electron donor rather than due to the presence of higher concentrations of chlorinated solvents. Because 90% VC dechlorination occurred following the second biostimulation event, it is likely that a single biostimulation event was not successful in stimulating dechlorination because of increased competition for electron donor in microcosms previously incubated at 50 an 70°C. Interestingly, a second biostimulation event was not required in autoclaved microcosms, suggesting that competition was caused by native organisms rather than populations introduced with the bioaugmentation inoculum. In fact, metabolic activity by native organisms was observed in the Ft. Lewis

microcosms previously incubated at 70°C during cooling and previous studies have demonstrated that microbes may rapidly recover following thermal remediation (31-32).

Dechlorination was incomplete in the unautoclaved Great Lakes microcosms previously incubated at 70°C even during repeated biostimulation. Because fewer electron equivlents were consumed in these microcosms than in microcosms in which complete dechlorination occurred, this lack of complete dechlorination in microcosms previously incubated at 70°C was not due to higher concentrations of chlorinated ethenes. The incomplete dechlorination coupled to the addition of significant electron donor suggests that inhibitory conditions were present in the unautoclaved microcosms previously incubated at 70°C.

Microcosms constructed from soils collected from both the Ft. Lewis and Great Lakes sites demonstrated limited dechlorination activity following cooling from 70°C. Hence, bioaugmentation following *in situ* thermal treatment may be less effective in regions of the thermal plume that were maintained near 70°C. Interestingly, in a previous study, a bioaugmented and biostimulated microcosm that was previously unheated dechlorinated TCE to VC and ethene whereas a microcosm that had previously been heated to 100°C only dechlorinated TCE to *cis*-DCE (15). These results confirm that exposing soils to elevated temperatures may decrease dechlorination activity by increasing the success of competitors or, in some cases, by inhibiting dechlorination activity. Even so, in the current study, in almost all microcosms, bioaugmentation and biostimulation fueled complete, rapid reductive dechlorination to ethene.

3.5.6 Competition for Electron Donor

Following bioaugmentation alone, more CH_4 was produced in unautoclaved Ft. Lewis microcosms previously incubated at 24 and 35°C than in autoclaved microcosms previously incubated at the same temperatures, suggesting that methanogens were present in the soil. Likely, the electron donor provided in the inoculum stimulated methanogenic activity in these microcosms. Similarly, Friis et al. observed that more CH_4 was generally produced in unheated microcosms compared to those that had been incubated at 100°C prior to bioaugmentation and biostimulation (15). These results support the suggestion that exposure to elevated temperatures decreases competition for electron donor from native microbes. Even so, following bioaugmentation alone in Ft. Lewis microcosms, CH_4 concentrations did not correlate with previous incubation temperature and, following 90% dechlorination, CH_4 concentrations positively correlated with previous incubation temperature. Therefore, although CH_4 production was initially inhibited due to incubation at elevated temperatures, more CH_4 was eventually produced in microcosms previously incubated at elevated temperatures, likely by methanogens introduced with the inocula. Similarly, following bioaugmentation in Great Lakes microcosms, more CH_4 was generally produced in microcosms previously incubated at higher temperatures. In the case of both Ft. Lewis and Great Lakes microsms, fewer electron equivalents were required for dechlorination in microcosms previously incubated at higher temperatures, but the time required for dechlorination did not correlate with previous incubation temperature. These results suggest that the higher levels of CH_4 in microcosms previously incubated at elevated temperatures were produced at the expense of reductive dechlorination.

Following bioaugmentation of Great Lakes microcosms, dechlorination extents ranged from 57 to 74% and, because nearly all PCE was converted to VC prior to ethene production, the dominant product of dechlorination in almost all microcosms was VC. CH_4 concentrations were highest in microcosms with the highest VC concentrations, or those microcosms in which nearly only VC remained. These results support the suggestion that VC-dechlorinating organisms could not compete successfully for reducing equivalents, even when electron donor was released due to heating.

3.6 Conclusions

The results of the current study demonstrate:

- Dechlorination activity may be limited even in the outer regions of the thermal plume where temperatures are not inhibitory to dechlorinating populations.
- Although thermal treatment increases electron donor bioavailability, the majority of reducing equivalents may be consumed in competing microbial processes**.**
- Bioaugmentation with cultures that do not contain methanogens may increase the efficiency of H_2 consumption for the microbial reductive dechlorination process.
- The exposure of soils to elevated temperatures may decrease dechlorination activity even once soils have cooled.

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CHAPTER 4

RESOLUTION OF CULTURE *CLOSTRIDIUM BIFERMENTANS* DPH-1 INTO TWO POPULATIONS: A *CLOSTRIDIUM* SP. AND TETRACHLOROETHENE (PCE) DECHLORINATING *DESULFITOBACTERIUM HAFNIENSE* STRAIN JH1

4.1 Abstract

Clostridium bifermentans strain DPH-1 reportedly dechlorinates tetrachloroethene (PCE) to *cis*-1,2-dichloroethene (*cis*-DCE). Cultivation-based approaches resolved culture DPH-1 into two populations: a non-dechlorinating *Clostridium* sp. and PCE dechlorinating *Desulfitobacterium hafniense* strain JH1. Strain JH1 carries *pceA* encoding a PCE reductive dehalogenase and shares other characteristics with *Desulfitobacterium hafniense* strain Y51.

4.2 Introduction

A variety of bacteria including *Dehalobacter*, *Desulfitobacterium*, *Desulfuromonas*, *Geobacter*, and *Sulfurospirillum* spp. reductively dechlorinate the

groundwater contaminants tetrachloroethene (PCE) and trichloroethene (TCE) to *cis*-1,2-dichloroethene (*cis*-DCE) (1-5). These organisms belong to the delta/epsilon subdivisions of the Proteobacteria and the Firmicutes. Among the Firmicutes, all PCE dechlorinating strains belong to the genera *Dehalobacter* and *Desulfitobacterium* with one exception: the spore-forming *Clostridium bifermentans* strain DPH-1 (6). Spore-forming PCE dechlorinators may play relevant roles for initiating dechlorination following exposure to unfavorable conditions during physical-chemical remediation, including thermal treatment of PCE/TCE source zones. In this study, we report the resolution of culture DPH-1 into two populations, a non-dechlorinating *Clostridium bifermentans* strain and a non-sporulating, PCE-dechlorinating *Desulfitobacterium hafniense* strain designated JH1.

4.3 Materials and Methods and Results

4.3.1 Resolution of Culture DPH-1 into Two Populations

Culture DPH-1 was maintained in 160-mL (nominal capacity) serum bottles containing 100 mL anoxic, reduced, bicarbonate-buffered (30 mM) mineral salts medium (4) amended with acetate (5 mM), citrate (5 mM), yeast extract (2 g/L), and PCE (240 μM, aqueous concentration). All cultures received 3% (vol/vol) inocula and were incubated at 24°C without agitation in the dark. Under these conditions, culture DPH-1 reduced PCE to stoichiometric amounts of *cis*-DCE with the intermediate formation of TCE in 10 to 20 days, and this activity was stable upon repeated transfers. When culture fluid was spread on Luria Bertani (LB) agar plates, uniform colonies formed within 1

week of incubation inside an anoxic chamber (95% nitrogen/5% hydrogen, vol/vol). Unexpectedly, when cells from isolated colonies were transferred to liquid medium, PCE dechlorination activity was not recovered after more than 6 months of incubation although visible growth occurred within 1 day (Figure 4.1).

In order to isolate the organism responsible for PCE dechlorination, two sequential dilution-to-extinction series were performed in 20-mL (nominal capacity) vials containing 9 mL of mineral salts medium amended with acetate (5 mM), H_2 (10% headspace volume), and PCE (2.5 µl) dissolved in hexadecane (47.5 µl) to yield an initial aqueous phase PCE concentration of approximately 460 µM (7). Dechlorination of PCE to *cis*-DCE occurred in the 10^{-10}-dilution vial, which served as the source for the second dilution-to-extinction series. Dechlorination activity occurred in the 10^{-9}-dilution vial, but when aliquots from this culture were spread onto LB agar plates, no colonies formed. Microscopic analysis corroborated the presence of two distinct organisms in culture DPH-1. Slender rods were observed in the 10^{-9}-dilution vial whereas the dominant organism in the original DPH-1 culture was a short, thick rod.

Phylogenetic analysis confirmed the presence of two populations in culture DPH-1. Genomic DNA was extracted from the non-dechlorinating isolate obtained following clonal purification on agar plates and 16S rRNA genes were PCR-amplified using bacterial primers 8F and 1525R as described (8). The 16S rRNA gene amplicons were cloned, and four cloned fragments were sequenced (8). The four sequences (>98.9% similarity) yielded a 1,369-bp 16S rRNA consensus gene sequence that was 97.3% similar to the reported *Clostridium bifermentans* strain DPH-1 16S rRNA gene sequence (GenBank accession number Y18787.1). Alignment of the reported DPH-1 16S rRNA

gene sequence with sequences from the non-dechlorinating isolate and the 10 most closely related sequences (GenBank accession numbers AY587782.1, AY587781.1, EF052864.1, AY167932.1, DQ978211.1, DQ218319.1, AY587793.1, AY167941.1, AF320283.1, EF052865.1) demonstrated that the DPH-1 sequence included a 30-bp repeat from position 1068 to 1097 (*E. coli* numbering). The alignment revealed six additional mismatches between the reported DPH-1 16S rRNA gene sequence and the non-dechlorinating *Clostridium* isolate, which likely represent sequencing errors and/or sequence variability (9-10). Excluding the 30 bp repeat, the reported strain DPH-1 sequence and the sequence of the non-dechlorinating isolate share 99.5% identity. Genomic DNA was extracted from the dechlorinating pure culture obtained following serial dilutions and the 16S rRNA gene was amplified, cloned, and sequenced (8). The 16S rRNA gene sequence of the dechlorinating isolate was 99.6% similar (1,387 bp analyzed) to the 16S rRNA gene sequence of *Desulfitobacterium hafniense* strain Y51 (accession number AP008230.1), a known PCE-to-*cis*-DCE dechlorinating bacterium (11). PCR with *Desulfitobacterium* spp. 16S rRNA gene-targeted primers (12) yielded an amplicon diagnostic for *Desulfitobacterium* with template DNA from the dechlorinating DPH-1 culture (data not shown). Therefore, we propose that the organism responsible for PCE dechlorination in culture DPH-1 is a *Desulfitobacterium hafniense* strain, which was designated strain JH1.

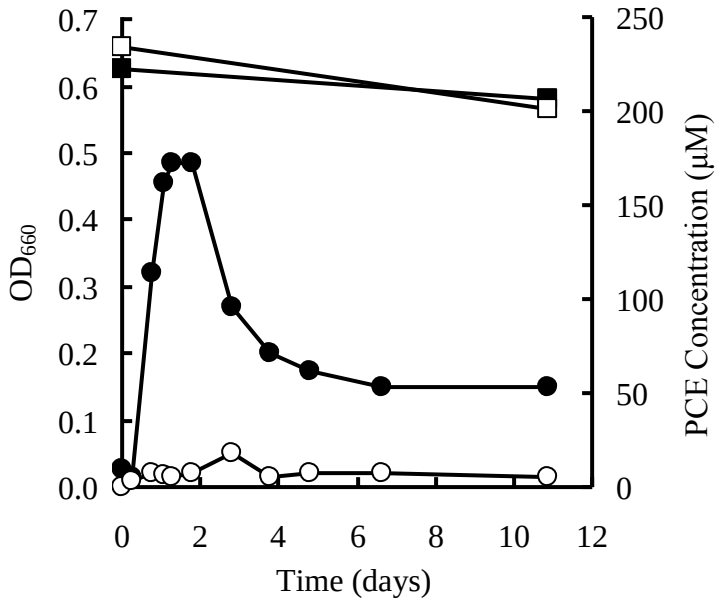

Figure 4.1 Growth in liquid cultures inoculated with isolated colonies from LB agar plates. Optical density (circles) and PCE concentration (squares) were monitored in live cultures (closed symbols) and cell-free controls (open symbols). Data were averaged from duplicate cultures. Average variability between data points was 26.8%.

4.3.2 Physiological Characterization of Strain JH1

Electron acceptor utilization was tested in 60-mL (nominal capacity) serum bottles containing 30 mL anoxic, reduced, bicarbonate-buffered mineral salts medium amended with 5 mM pyruvate, which supported fermentative growth and served as electron donor and carbon source. Cultures were amended with undiluted chloroethanes, chloroethenes, chloromethanes, chloropropanes, or 2-chlorotoluene using a gas-tight Hamilton syringe (1800 series; Hamilton, Reno, NV) to yield final aqueous concentrations ranging from 100 to 250 μM. Chlorinated aliphatic compounds and 2-chlorotoluene were analyzed by gas chromatography as described (13). Hexachlorobenzene was added using a Hamilton syringe from a methanolic stock to an aqueous concentration of 0.09 μM and analyzed by liquid/liquid extraction in hexane, followed by gas chromatographic separation, and detection using an electron capture detector. 3-Chloro-4-hydroxybenzoate, nitrate, sulfate, and sulfite were added from anoxic, sterile, aqueous stock solutions using plastic syringes to final concentrations of 1 to 2 mM. 3-Chloro-4-hydroxybenzoate was analyzed as described (14) and inorganic anions were analyzed with a Dionex ICS-3000 ion chromatograph equipped with an AS14 4-mm column (Dionex, Sunnyvale, CA). Soluble Fe(III) (as Fe(III) citrate) and poorly crystalline Fe(III) oxide were prepared as described (4) and added at 5 mM (nominal) concentrations. Fe(II), total iron, ammonia, and sulfide concentrations were determined colorimetrically (15-16).

Cultures of strain JH1 completely reduced (products given in brackets) PCE [*cis*-DCE] (Figure 4.2), TCE [*cis*-DCE], nitrate [ammonium], sulfite [sulfide], soluble Fe(III) [Fe(II)], and poorly crystalline Fe(III) oxide [Fe(II)]. Cultures amended with 1,1,2,2-

tetrachloroethane formed 1,1,2-trichloroethane (22% mol/mol), *cis*-DCE (57%), and *trans*-DCE (21%). Under the conditions tested, strain JH1 did not reduce 1,1,2-trichloroethane, 1,1-dichloroethane (1,1-DCA), 1,2-DCA, *cis*-DCE, *trans*-DCE, vinyl chloride, carbon tetrachloride, chloroform, dichloromethane, 1,2,3-trichloropropane, 1,2-dichloropropane, 2-chlorotoluene, hexachlorobenzene, 3-chloro-4-hydroxybenzoate, or sulfate.

To test electron donor utilization, culture vessels were amended with 100 μM PCE and inocula (3%, vol/vol) from a culture that had consumed all pyruvate. Once PCE dechlorination ceased due to electron donor limitation, cultures were amended with potential electron donors including acetate (5 mM), ethanol (170 μM), formate (5 mM), or H_2 (10% headspace volume). Strain JH1 used formate, ethanol, and H_2 as electron donors, but acetate did not support reductive dechlorination under the conditions tested. Some *Desulfitobacterium* spp. have been reported to form spores (17), but repeated efforts to recover activity from stationary phase strain JH1 cultures exposed to temperatures ranging from 60-80°C for 10 minutes (18) were not successful.

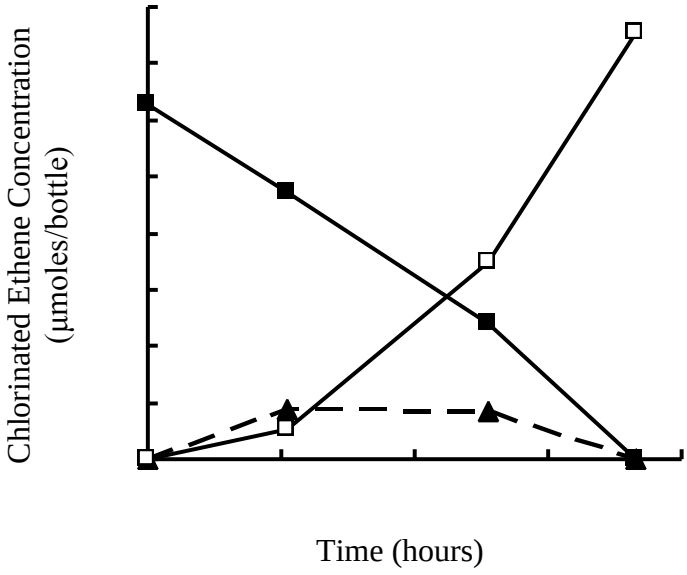

Figure 4.2 Dechlorination of PCE (filled squares) to *cis*-DCE (open squares) with the intermediate formation of TCE (triangles) by strain JH1. Data were averaged from duplicate cultures. Average variability between data points was 35.0%.

4.3.3 Identification of the PCE Reductive Dehalogenase (RDase) Gene

The PCE RDase genes *pceC* (AJ277528) and *pceA* (AP008230.1) have been identified in culture DPH-1 (19) and *Desulfitobacterium hafniense* strain Y51 (20), respectively. To amplify the *pceC* gene reported in culture DPH-1, primers (pceCF, 5'-CGGTCATCAGAGAAATAATG and pceCR, 5'-GCTGAAGTTTATAATAAAGA) were designed based on published degenerate primers (19). Genomic DNA from the mixed DPH-1 culture, strain JH1, and the *Clostridium* isolate served as templates in separate PCR reactions (19); however, none of the assays yielded the expected 81-bp amplicon over the range of annealing temperatures (32.6 to 41.6ºC), $MgCl_2$ concentrations (2.5 to 4.0 mM), and number of PCR cycles (30 to 40) tested. To amplify the *pceA* gene reported in strain Y51, primers (pceAF, 5'-CGGACATCGTGGCTCCGAT and pceAR, 5'-CTTGTCCGGAGCAAGTTC) were designed based on the degenerate primers reported previously (20). PCR reactions were carried out as described (8), but at an annealing temperature of 46.5ºC. Amplicons of the expected size (1,000 bp) were obtained with genomic DNA from culture DPH-1 and strain JH1, whereas genomic DNA from the *Clostridium* isolate did not yield a visible amplification product in ethidium bromide-stained agarose gels. The amplicons were purified (Qiagen QIAquick PCR Purification Kit, Germantown, MD) and sequenced using primers pceAF and pceAR. The sequence of the 935-bp fragment exactly matched the reported Y51 *pceA* gene sequence (20).

4.4 Discussion

Desulfitobacterium hafniense strain JH1 shares many physiological properties with strain Y51, but, in contrast to strain Y51, dechlorinated 1,1,2,2-tetrachloroethane to a mixture of 1,1,2-trichloroethane, *cis*-DCE, and *trans*-DCE rather than only to *cis*-DCE and used ethanol as electron donor. Consistent with *Desulfitobacterium* physiology, strain JH1 failed to reduce sulfate whereas strain Y51 reportedly reduced sulfate (11).

Co-enrichment of PCE-dechlorinators with *Clostridium* spp. is not unprecedented. For example, Sung et al. (5) reported a co-culture consisting of the PCE dechlorinator *Desulfuromonas michiganensis* strain BB1 and *Clostridium sphenoides*. Hence, unexplored, possibly symbiotic, nutritional interactions between *Clostridium* spp. and dechlorinators may exist. Understanding the interactions between dechlorinators and non-dechlorinating populations is relevant for successful bioremediation, emphasizing the need for detailed studies of the ecology of bacteria capable of respiratory reductive dechlorination (i.e., [de]chlororespiration).

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CHAPTER 5

EFFECTS OF ELEVATED TEMPERATURE ON *DEHALOCOCCOIDES* DECHLORINATION PERFORMANCE AND DNA AND RNA BIOMARKER ABUNDANCE

Reproduced in part with permission from Fletcher, K. E.; Costanza, J.; Cruz-Garcia, C.; Ramaswamy, N. S.; Pennell, K. D.; Löffler, F. E. Effects of elevated temperature on *Dehalococcoides* dechlorination performance and DNA and RNA biomarker abundance. *Environ. Sci. Technol.* **2010**, submitted for publication.

5.1 Abstract

Coupling thermal treatment with microbial reductive dechlorination is a promising remedy for tetrachloroethene (PCE) and trichloroethene (TCE) contaminated source zones. Laboratory experiments evaluated *Dehalococcoides* (*Dhc*) dechlorination performance, viability, and biomarker gene (DNA) and transcript (mRNA) abundances during exposure to elevated temperatures. The PCE-dechlorinating consortia BDI and OW produced ethene when incubated at temperatures of 30°C, but vinyl chloride (VC) accumulated when cultures were incubated at 35 or 40°C. Cultures incubated at 40°C for less than 49 days resumed VC dechlorination following cooling; however, incubation at 45°C resulted in complete loss of dechlorination activity. *Dhc* 16S rRNA, *bvcA*, and *vcrA* gene abundances in cultures showing complete dechlorination to ethene at 30°C exceeded those measured in cultures incubated at higher temperatures, consistent with observed dechlorination activities. Conversely, biomarker gene transcript abundances in

cultures incubated at 35 and 40°C were generally at least one order-of-magnitude greater than those measured in ethene-producing cultures incubated at 30°C. Even in cultures accumulating VC, transcription of the *vcrA* gene, which is implicated in VC-to-ethene dechlorination, was up-regulated. Apparently, temperature stress caused the up-regulation of *Dhc* reductive dehalogenase gene expression indicating that *Dhc* gene expression measurements should be interpreted cautiously as *Dhc* biomarker gene transcript abundances may not correlate with dechlorination activity.

5.2 Introduction

The chlorinated solvents PCE and TCE are often present as dense non-aqueous phase liquids (DNAPLs), which may serve as long-term sources of groundwater contamination (1). Thermal treatment removes significant amounts of PCE and TCE from source zones, but complete contaminant removal is unlikely to be achieved (2-3). Therefore, thermal treatment coupled to bioremediation has been suggested as a promising remediation approach (4-5). During thermal treatment, the source zone is heated to temperatures approaching 100°C or greater (6) and subsurface temperatures may remain elevated for prolonged periods following heating unit shutdown (7).

Anaerobic bioremediation of chlorinated ethenes relies on sequential reductive dechlorination where PCE is transformed via the intermediates TCE, dichloroethenes (DCEs), and vinyl chloride (VC) to yield ethene and inorganic chloride. Although numerous bacteria are capable of PCE and TCE dechlorination to DCEs, only some members of the *Dehalococcoides* (*Dhc*) group are known to reductively dechlorinate

DCEs and VC to ethene (8). Metabolic microbial reductive dechlorination of chlorinated ethenes to ethene has been reported to occur at temperatures ranging from 10 to 30°C, and is therefore considered to be a mesophilic process (7, 9-10). Even so, because subsurface temperatures will decrease with distance away from the heated treatment zone, dechlorination may occur in the perimeter of the heated zone even during treatment. Similarly, dechlorination may occur in the treated source zone following cooling, although it is unknown if dechlorinating populations will recover activity following exposure to elevated temperatures.

Molecular techniques targeting *Dhc* biomarkers (e.g., the *Dhc* 16S rRNA gene and the *bvcA, tceA,* and *vcrA* reductive dehalogenase [RDase] genes and gene transcripts) are used to assess *Dhc* abundance and activity (11-19). The quantity of *Dhc* biomarker genes has been correlated with dechlorination activity in both laboratory and field studies (11-12, 20-23); however, measurements of *Dhc* biomarker gene abundances do not differentiate viable, dechlorinating cells from inactive or nonviable cells (18, 24). Gene expression (i.e., transcription), quantified by analysis of cDNA, is generally considered to be a more direct measurement of cellular activity (8, 24). Previous studies have suggested that *Dhc* biomarker gene transcript abundances correspond to the metabolic state of *Dhc* populations (13, 18, 20, 24-28). For example, Lee et al. (2006) demonstrated that *tceA* gene expression was 90-fold greater in *Dhc* cultures exposed to TCE than in chlorinated ethene-free controls (18). Conversely, Amos et al. (2008) detected *tceA* and *vcrA* gene transcripts in oxygen exposed, non-dechlorinating *Dhc* cultures and demonstrated that biomarker gene transcription did not always correlate with dechlorination (in)activity (24).

The consequences of elevated temperatures on *Dhc* activity as well as biomarker gene and gene transcript abundances are poorly understood. More complete knowledge of *Dhc* response to and activity during and following exposure to elevated temperatures is relevant for predicting the success of coupling thermal treatment with microbial reductive dechlorination. Therefore, the goals of this study were to determine i) the effects of elevated temperatures on *Dhc* dechlorination activity, ii) if contemporary nucleic acid-based tools accurately assess *Dhc* activity during exposure to elevated temperatures, and iii) if *Dhc* dechlorination activity has the potential to recover following exposure to elevated temperatures.

5.3 Materials and Methods

5.3.1 Cultures and Medium Preparation

Two PCE-to-ethene dechlorinating mixed cultures, Bio-Dechlor INOCULUM (BDI) and the OW consortium, were evaluated in this study. BDI is a non-methanogenic, PCE-to-ethene dechlorinating consortium that has been successfully applied for bioremediation at chlorinated solvent contaminated sites (29). BDI contains *Dhc* strain BAV1, strain FL2, and strain GT (17), which contain the *bvcA*, *tceA*, and *vcrA* genes, respectively (14, 30-31). Strain BAV1 metabolically dechlorinates DCEs to ethene, strain FL2 metabolically dechlorinates TCE to VC, and strain GT metabolically dechlorinates TCE to ethene (30-32). BDI also contains a PCE-to-*cis*-DCE-dechlorinating *Dehalobacter* strain (33-34). OW is a methanogenic PCE-to-ethene dechlorinating consortium that contains multiple *Dhc* strains carrying the *tceA* and *vcrA*

genes (35). The OW consortium also contains 16S rRNA gene sequences similar to *Dehalobacter*, *Geobacter*, and *Sulfurospirillum* strains known to reduce PCE to *cis*-DCE (34-37).

Reduced, anaerobic, bicarbonate-buffered (30 mM) mineral salts medium was prepared as described (33), except that N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid was omitted and the medium contained 0.2 mM Na_2S x 9 H_2O and only 0.4 mM DL-dithiothreitol. Medium for the OW consortium did not contain DL-dithiothreitol. Cultures were maintained in 160-mL (nominal capacity) glass serum bottles containing approximately 100 mL of medium and a N_2/CO_2 (80%/20%, vol/vol) headspace. Vitamins (38) and 5 mM lactate were added to sterile medium from anoxic stock solutions, and 100 µL of either a PCE- or TCE-methanol stock was added to yield final aqueous chlorinated ethene concentrations below 40 mg/L. Triplicate or duplicate cultures were incubated upside-down in the dark, under static conditions.

5.3.2 Dechlorination Activity During and Following Exposure to Elevated Temperatures

To assess reductive dechlorination as a function of incubation temperature, PCE- or TCE-amended cultures were incubated at temperatures of 30, 35, 40, and 45ºC. In experiments with the BDI consortium, bottles containing anaerobic, sterile, mineral salts medium were inoculated (3%, vol/vol), amended with PCE in methanol, and incubated at 24°C. Once all PCE was converted to ethene, cultures received additional PCE or TCE in methanol and were incubated at 30, 35, 40, or 45ºC. PCE-free control cultures (i.e., starved controls) were not amended with PCE or TCE and were incubated at 30°C. In experiments conducted with the OW consortium, bottles containing mineral salts medium

were inoculated (10%, vol/vol), amended with PCE in methanol, and immediately incubated at 30, 35, 40, and 45°C. To determine if *Dhc* populations are capable of recovering reductive dechlorination activity following exposure to elevated temperatures, triplicate BDI cultures were incubated at 24ºC following 7, 14, 28, and 49 days of incubation at 40°C.

5.3.3 Nucleic Acid Extraction and Quantitative Real-Time PCR (qPCR) Analysis

Aqueous samples (5 mL) were removed from BDI cultures and replaced with equivalent volumes of sterile mineral salts medium. Biomass was collected via centrifugation at 10,000 rpm for 10 minutes and RNAprotect Bacteria Reagent (Qiagen, Valencia, CA) was used to stabilize RNA (24). Pellets were stored at -80ºC prior to DNA and RNA extraction using the Allprep DNA/RNA Mini Kit (Qiagen) (24). To remove DNA from RNA, the RNA fraction was DNase treated with Baseline-Zero DNase (Epicentre Biotechnologies, Madison, WI) following the manufacturer's instructions. DNase was inactivated by the addition of 1.1 µL of 0.5 M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich Co., St. Louis, MO) and incubation at 70°C for 5 minutes. RNA was then purified using the RNeasy MinElute Cleanup Kit (Qiagen). In almost all cases, no amplicons were obtained in PCR reactions with RNA templates and universal bacterial 16S rRNA gene-targeted primers, demonstrating that DNA had been effectively removed. In the two RNA samples that yielded amplicons (i.e., samples contaminated with DNA), a second DNase treatment removed the remaining DNA. The volume of RNA solution was reduced to less than 8 µl using a SC210A SpeedVac Plus (ThermoSavant, Milford, MA) and RNA was reverse

transcribed into cDNA using the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

The *Dhc* 16S rRNA gene and the *bvcA, tceA,* and *vcrA* RDase genes were quantified in DNA and cDNA using an ABI 7500 Fast Real-Time PCR System (17, 24). Each *Dhc* cell in the BDI culture harbors a single copy of the 16S rRNA gene and one copy of either the *bvcA, tceA,* or *vcrA* gene (17, 24).

5.3.4 Analytical Methods

Aqueous samples (1 mL) were collected to quantify chlorinated ethenes and ethene as described (39), and gaseous samples (2 mL) were collected to quantify hydrogen. Removed aqueous and gaseous volumes were replaced with equal volumes of sterile mineral salts medium and sterile N_2 gas, respectively. Hydrogen concentrations were measured using a Hewlett Packard 6890 GC equipped with a heated gas sampling valve, a 250 µL sample loop, and a 30 m by 0.32 mm OD Carboxen-1010 column (Supelco, Bellefonte, PA) connected to a thermal conductivity detector.

5.4 Results

5.4.1 Reductive Dechlorination at Elevated Temperatures

In BDI cultures amended with PCE and incubated at 30ºC, PCE was stoichiometrically dechlorinated to ethene within 30 days (Table 5.1, Figure 5.1 A). In cultures incubated at 35ºC, PCE was dechlorinated to VC and ethene; however, even after 42 days, VC persisted and accounted for 27.3 ± 13.3% (mol/mol) of dechlorination

products (Table 5.1, Figure 5.1 B). During incubation at 40ºC, 24.9 ± 4.8% (mol/mol) of PCE was dechlorinated to VC (Table 5.1), but dechlorination ceased 2 days after PCE amendment (data not shown). For the remainder of the 96-day incubation period, PCE and VC persisted and no ethene production occurred. Hydrogen concentrations in cultures incubated at 40°C exceeded 1,000 ppmv, indicating that the cessation of dechlorination activity was not due to electron donor limitations. Interestingly, in BDI cultures amended with TCE and incubated at 40°C, all TCE was dechlorinated to 53.9 ± 13.9% (mol/mol) VC and 46.1 ± 13.9% (mol/mol) ethene (Table 5.1), demonstrating that the PCE-to-TCE and VC-to-ethene dechlorination steps are more sensitive to elevated temperatures than TCE and *cis*-DCE dechlorination. In cultures incubated at 45°C, 37.1 ± 2.6% (mol/mol) of TCE was dechlorinated to *cis*-DCE, VC, and ethene (Table 5.1), but dechlorination ceased 1 day after TCE amendment (data not shown).

In OW cultures incubated at 30°C, PCE was stoichiometrically converted to ethene, but incubation at 35 and 40ºC resulted in stoichiometric conversion of PCE to VC (Table 5.1). During incubation at 45ºC, only 6.7 ± 0.1% (mol/mol) of PCE was dechlorinated to 2.4 ± 0.1% (mol/mol) TCE and 4.3 ± 0.1% (mol/mol) VC, and no ethene was formed (Table 5.1). Therefore, experiments performed with the OW consortium confirmed that dechlorination of VC is more susceptible to elevated temperatures than TCE and *cis*-DCE dechlorination.

Table 5.1 Chlorinated ethenes and ethene measured in duplicate or triplicate consortia BDI and OW cultures incubated at 30, 35, 40, and 45°C. Following incubation of the BDI and OW cultures for 42 and 30 days, respectively, stable distribution patterns of chlorinated ethenes and ethene were measured.

Consortium	Electron Acceptor	Chlorinated Ethenes and Ethene (mol%)			
		30ºC	35ºC	40ºC	45ºC
BDI	PCE	Ethene (100%)	VC (27%) Ethene (73%)	PCE (75%) VC (25%)	Not tested
BDI	TCE	Not tested	Not tested	VC (54%) Ethene (46%)	TCE (63%) *cis*-DCE (18%) VC (5%) Ethene (15%)
OW	PCE	Ethene (100%)	VC (100%)	VC (100%)	PCE (93%) TCE (2%) VC (4%)

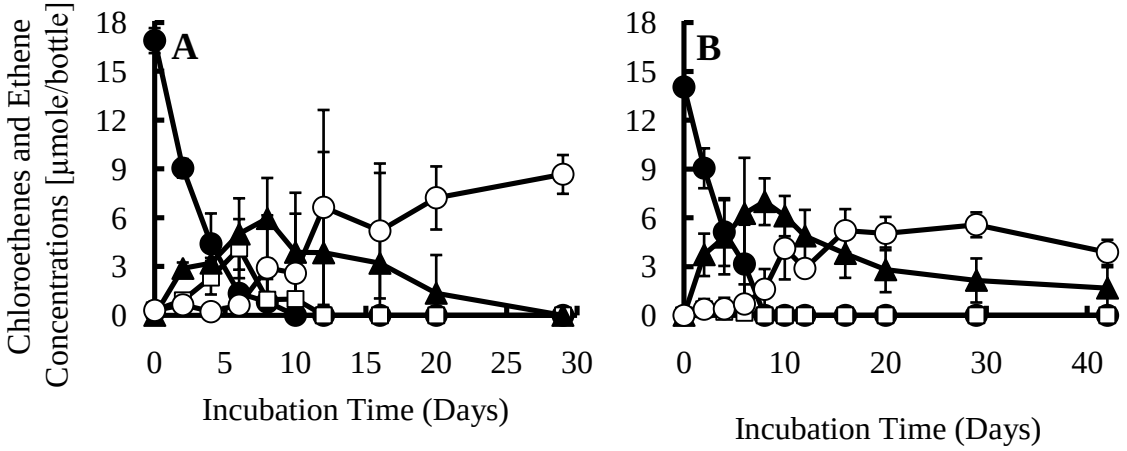

Figure 5.1 Dechlorination of PCE in BDI cultures incubated at 30 (A) and 35°C (B). PCE (solid circles) dechlorination to *cis*-DCE (open squares), VC (solid triangles), and ethene (open circles). TCE is not shown as less than 1 µmole/bottle TCE was formed. All data points represent average values from duplicate or triplicate cultures and error bars depict one standard deviation.

5.4.2 *Dhc* Biomarker Gene Abundances at Elevated Temperatures

Prior to incubation at elevated temperatures, $1.8 \times 10^8 \pm 6.4 \times 10^7$ *Dhc* 16S rRNA gene copies per mL were present in BDI cultures. In cultures incubated at 30°C for 42 days, the quantity of *Dhc* 16S rRNA genes increased to $2.5 \times 10^8 \pm 8.2 \times 10^7$ copies per mL. After 42 days of incubation, *Dhc* 16S rRNA genes were detected in cultures incubated at 35 and 40°C at a maximum abundance of $1.6 \times 10^8 \pm 2.4 \times 10^6$ copies per mL, approximately 65% of the quantity measured in cultures incubated at 30°C (Figure 5.2 A). After 42 days, strain FL2 cell titers were slightly higher in cultures incubated at 35°C than in those at 30°C, $6.8 \times 10^7 \pm 3.6 \times 10^7$ versus $3.2 \times 10^7 \pm 2.5 \times 10^6$ copies per mL, respectively. In cultures incubated at 40°C, $4.0 \times 10^6 \pm 1.4 \times 10^6$ strain FL2 cells per mL were detected, only 13% of the quantity in cultures incubated at 30°C and more than an order-of-magnitude fewer than were measured initially, $7.0 \times 10^7 \pm 2.9 \times 10^7$ (Figure 5.2 B).

Dhc strain BAV1 cells, quantified by *bvcA* gene copies, were detected at $9.9 \times 10^4 \pm 1.7 \times 10^4$ per mL prior to incubation at elevated temperatures. Only $6.6 \times 10^4 \pm 6.4 \times 10^3$ *bvcA* copies per mL were detected in cultures incubated at 30°C for 42 days, while in cultures incubated at 35 and 40ºC and in the starved control cultures, *bvcA* genes were below the detection limit (9.5×10^3 copies per mL culture fluid) (Figure 5.2 C). Strain GT cell titers, measured by *vcrA* quantification, increased slightly from $1.5 \times 10^8 \pm 3.2 \times 10^7$ to $2.1 \times 10^8 \pm 3.4 \times 10^7$ per mL after 42 days of incubation at 30°C. In cultures incubated at 35 and 40°C for 42 days, strain GT cell titers were $8.9 \times 10^7 \pm 2.0 \times 10^7$ and $7.3 \times 10^7 \pm 6.3 \times 10^6$ per mL, or only 42 and 34%, respectively, of the quantity detected

in cultures incubated at 30°C (Figure 5.2 D). Similar reductions in cell numbers were observed in the starved control cultures.

5.4.3 RDase Gene Transcript Abundances at Elevated Temperatures

From day 0 to day 6 of incubations at 35 and 40°C, the numbers of 16S rRNA molecules per cell increased by 0.97 ± 0.64 and 1.39 ± 0.61 orders-of-magnitude, respectively. Conversely, in cultures incubated at 30°C and in starved control cultures, the abundance of 16S rRNA molecules per cell decreased by nearly 2 orders-of-magnitude (Table 5.2). Therefore, the numbers of *Dhc* 16S rRNA molecules per *Dhc* cell were 2.9 ± 0.4 and 3.3 ± 0.3 orders-of-magnitude higher in cultures incubated at 35 and 40ºC, respectively, than in cultures incubated at 30ºC (Figure 5.3 A).

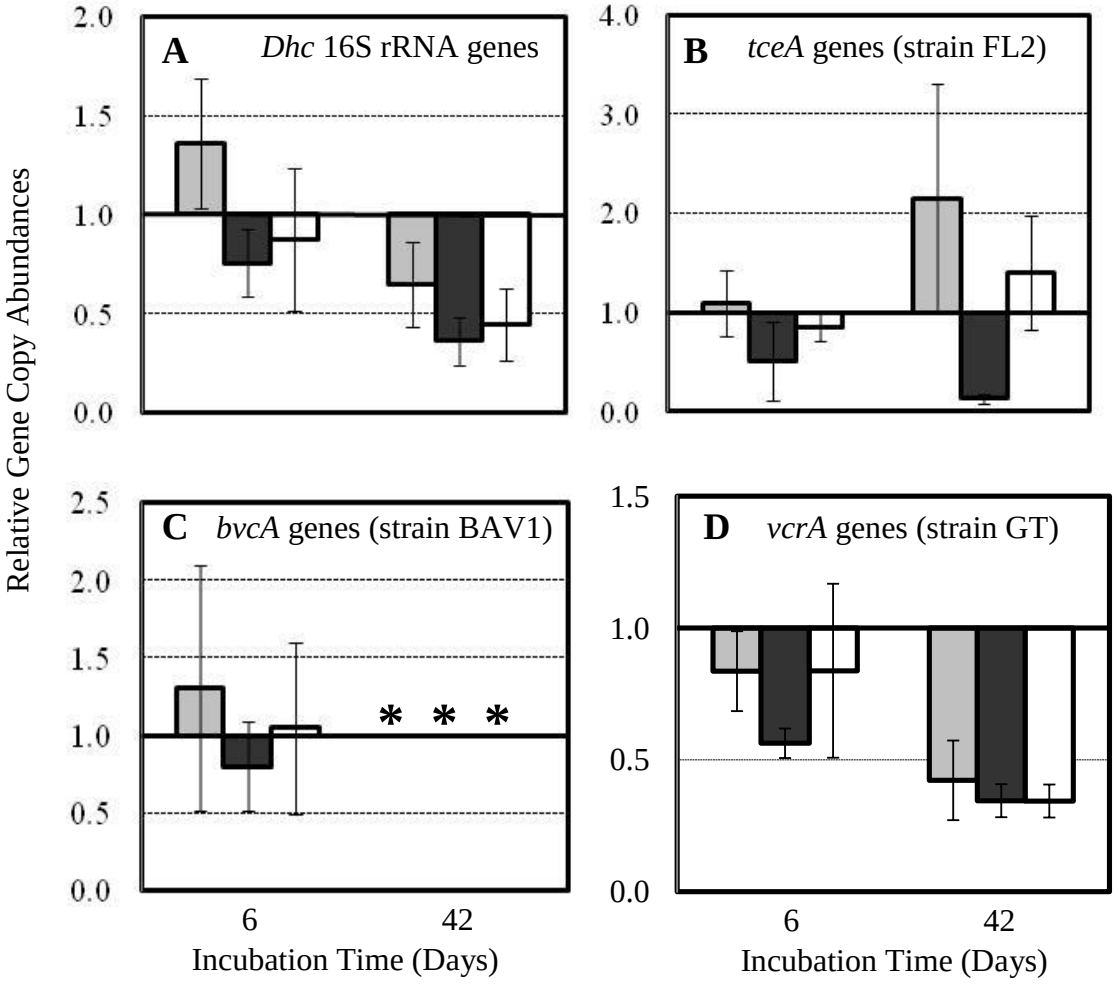

Figure 5.2 *Dhc* gene copy abundances in cultures incubated at 35ºC (gray bars), 40ºC (black bars), and in starved control cultures incubated at 30ºC (white bars). Abundances of *Dhc* 16S rRNA (A), *tceA* (B), *bvcA* (C), and *vcrA* (D) gene copies were normalized to quantities measured in cultures incubated at 30°C and amended with PCE. The asterisks indicate that the *bvcA* gene was below the detection limit of 9.5×10^3 gene copies per mL culture fluid in cultures incubated at 35 and 40°C and in starved control cultures after 42 days of incubation.

Table 5.2 16S rRNA molecule and RDase gene transcript abundances in duplicate or triplicate BDI cultures incubated at 30, 35, and 40°C and in starved control cultures incubated at 30°C. Abundances were normalized to 16S rRNA molecule and gene transcript quantities measured immediately prior to PCE amendment (i.e., time zero).

Culture	Log Transcripts per Cell Relative to Time Zero		
	***Dhc* 16S rRNA**	***tceA***	***vcrA***
6 Days of Incubation			
30°C	-1.95 ± 0.53	1.32 ± 1.00	-0.25 ± 0.84
35°C	0.97 ± 0.64	4.15 ± 1.06	2.40 ± 0.8
40°C	1.39 ± 0.61	1.86 ± 1.01	1.90 ± 0.83
Starved Control	-2.01 ± 0.65	-[a]	-0.83 ± 0.86
42 Days of Incubation			
30°C	1.31 ± 0.54	1.83 ± 0.99	0.92 ± 0.82
35°C	1.33 ± 0.54	1.41 ± 0.99	0.82 ± 0.79
40°C	1.37 ± 0.54	2.77 ± 1.21	0.79 ± 0.84
Starved Control	1.41 ± 0.55	1.49 ± 1.06	0.48 ± 0.76

[a] Transcripts were below the detection limit of 3.2×10^2 transcripts per mL of culture fluid.

In cultures incubated at 30, 35, and 40°C, the number of *tceA* gene transcripts per strain FL2 cell increased by more than an order-of-magnitude over a 6-day incubation period (Table 5.2); however, the number of *tceA* gene transcripts per strain FL2 cell was still 2.8 ± 0.4 orders-of-magnitude higher in cultures incubated at 35ºC than in cultures incubated at 30ºC after 6 days of incubation (Figure 5.3 B). In starved control cultures, *tceA* gene transcripts were below the detection limit of 3.2×10^2 transcripts per mL of culture. Transcripts of the *bvcA* gene were not detected in any culture at any time. Over a 6-day incubation period at 35 and 40°C, the number of *vcrA* gene transcripts per strain GT cell increased by at least one order-of-magnitude (Table 5.2). In cultures incubated at 30°C, *vcrA* gene transcript abundance remained approximately unchanged. The abundances of *vcrA* gene transcripts per strain GT cell in cultures incubated at 35 and 40°C were 2.7 ± 0.5 and 2.1 ± 0.5 orders-of-magnitude higher, respectively, than in cultures incubated at 30ºC (Figure 5.3 C).

In all cultures, the abundances of 16S rRNA molecules and *tceA* transcripts were over one order-of-magnitude greater after 42 days of incubation than immediately prior to PCE amendment (Table 5.2). After 42 days of incubation, the abundances of *vcrA* transcripts in all cultures were similar to initial quantities (Table 5.2). Therefore, after 42 days of incubation, the abundances of 16S rRNA molecules and *tceA* and *vcrA* transcripts in cultures incubated at 35 and 40°C and in starved control cultures were within an order-of-magnitude of the quantities in cultures incubated at 30°C (Figure 5.3).

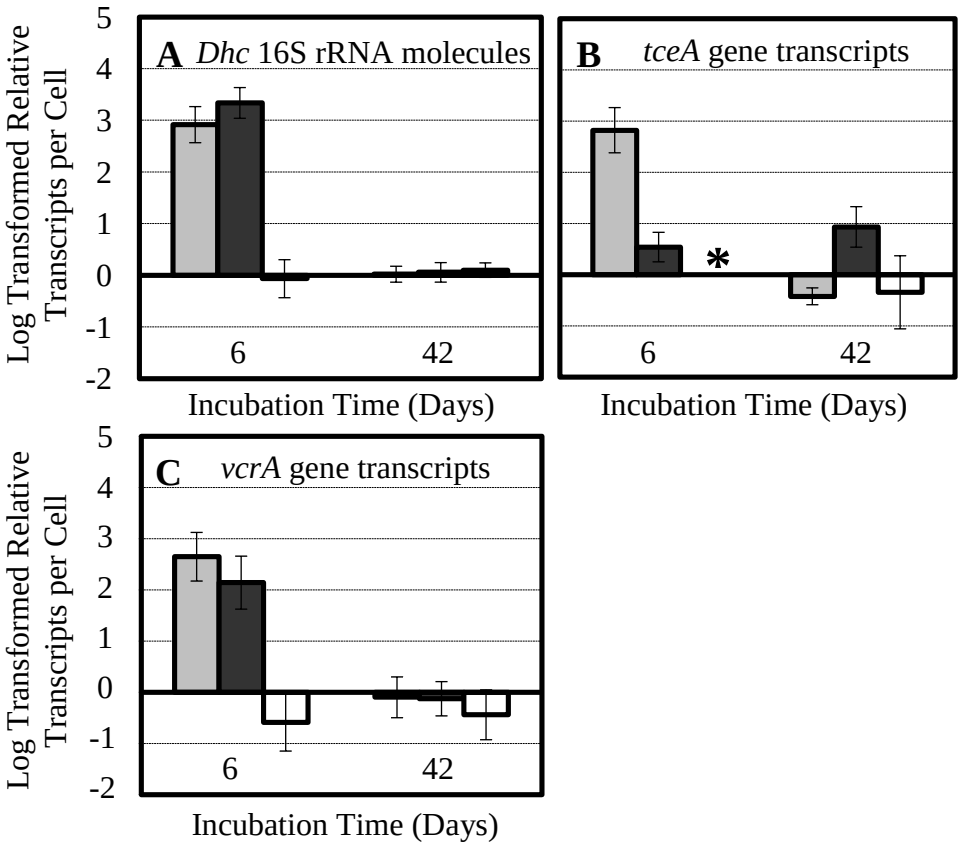

Figure 5.3 *Dhc* 16S rRNA molecule and RDase gene transcript abundances in cultures incubated at 35ºC (gray bars), 40ºC (black bars), and in starved control cultures incubated at 30ºC (white bars). Abundances of *Dhc* 16S rRNA (A), *tceA* (B), and *vcrA* (C) gene transcripts were normalized to quantities measured in cultures incubated at 30°C and amended with PCE. The relative abundances were log transformed. The asterisk indicates that *tceA* gene transcripts were below the detection limit of 3.2×10^2 transcripts per mL of culture fluid in the starved cultures after 6 days of incubation.

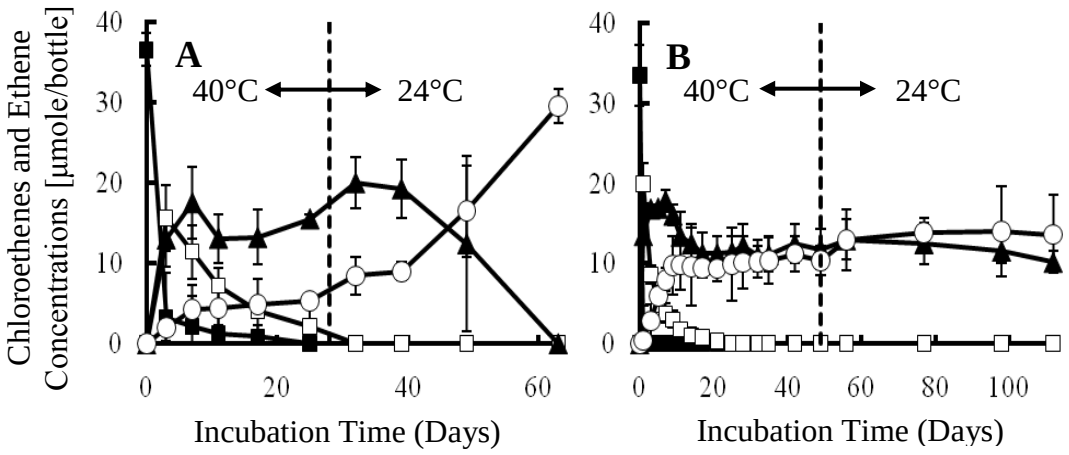

Figure 5.4 Dechlorination activity in BDI cultures incubated at 24°C following 28 (A) and 49 days (B) of incubation at 40°C. TCE (solid squares) dechlorination to *cis*-DCE (open squares), VC (solid triangles), and ethene (open circles) in the BDI cultures. The vertical dashed lines indicate the decrease in incubation temperature from 40 to 24ºC. All data points represent average values from triplicate cultures and error bars depict one standard deviation.

5.4.4 VC Dechlorination Activity Following Cooling

During incubation at 40ºC, BDI cultures dechlorinated TCE to VC. Prior to cooling, VC persisted and accounted for 58.0 ± 13.5% (mol/mol) of dechlorination products. Following cooling to 24ºC, cultures previously incubated at 40ºC for 7, 14, and 28 days completely dechlorinated VC in 21, 28, and 35 days, respectively (Figure 5.4 A). In cultures incubated at 40ºC for 49 days, VC was not dechlorinated to ethene after cooling to 24ºC (Figure 5.4 B).

5.5 Discussion

5.5.1 Effects of Elevated Temperatures on Dechlorination Activity

In BDI cultures, PCE dechlorination was inhibited at 40°C while TCE dechlorination was not. The BDI consortium contains a *Dehalobacter* population that is capable of PCE-to-*cis*-DCE dechlorination. The *Dehalobacter* population, as well as *Dhc* strain FL2 and strain GT, use TCE as a metabolic electron acceptor. While it is possible that the *Dehalobacter* sp. catalyzed the TCE-to-*cis*-DCE, but not the PCE-to-TCE dechlorination step at 40°C, the PceA enzyme in *Dehalobacter restrictus* has been shown to catalyze both reactions (40). Also, *D. restrictus* has been shown to be sensitive to temperatures above 35°C (34). Therefore, TCE dechlorination at 40°C was likely catalyzed by *Dhc* strain FL2 and/or strain GT present in consortium BDI. PCE dechlorination was not inhibited during incubation at 40°C in OW cultures, which contain chlorinated ethene-dechlorinating *Dhc*, *Dehalobacter*, *Geobacter*, and *Sulfurospirillum* populations (35). Characterized PCE-dechlorinating *Sulfurospirillum*

multivorans strains cannot grow at temperatures above 37°C (36), but metabolic activity of *Geobacter lovleyi* strain SZ has been demonstrated at 40°C (37), suggesting that this population contributed to PCE dechlorination in consortium OW incubated at 40°C. Temperatures of 35°C or greater inhibited dechlorination of VC to ethene by the *Dhc* strains present in both the BDI and OW consortia. In BDI cultures, dechlorination activity recovered when cultures were cooled from 40°C to 24°C and the incubation time at 40°C did not exceed 24 days. Longer incubation times resulted in loss of VC dechlorination activity, suggesting that extended incubation at 40°C irreversibly inactivated VC-dechlorinating *Dhc* strains. Interestingly, TCE-to-VC dechlorination was not inhibited at 40°C (Table 5.1). In a previous study, TCE-to-VC dechlorination recovered following a 30-day exposure to ≤ 4 mg/L of dissolved oxygen, but VC dechlorination was irreversibly inhibited (24). These observations suggest that reductive VC dechlorination is more susceptible to inhibition by elevated temperatures and oxygen exposure than the TCE and *cis*-DCE dechlorination steps.

During thermal treatment, temperature gradients will develop, with subsurface temperatures decreasing away from the heated treatment zone. The results from the current study demonstrate that i) when temperatures are 35°C or greater, complete dechlorination to ethene is unlikely and ii) in subsurface zones with temperatures between 35°C and 45°C, *Dhc* populations may contribute to the formation of VC, a known human carcinogen (41). Our results show that elevated temperatures have distinct effects on individual steps of chlorinated ethene dechlorination, even the *cis*-DCE-to-VC and VC-to-ethene dechlorination steps that are catalyzed exclusively by *Dhc* populations. In a previous study with consortium KB-1, *cis*-DCE rather than VC accumulated during

incubation at 40°C (7), suggesting that the *cis*-DCE-to-VC step is more sensitive to elevated temperatures in KB-1 than in BDI. Only a single report describes the dechlorination of chlorinated ethenes by a thermophile (an organism with an optimal growth temperature greater than 45°C) (42). A *Dehalobacter* sp. in an enrichment culture derived from sediments from the harbor of Rotterdam, The Netherlands was implicated in PCE-to-*cis*-DCE dechlorination at 65°C (43). Some (partially) purified RDases have been shown to maintain dechlorination activity at temperatures of 50 - 60°C (44-46), suggesting that biologically-catalyzed reductive dechlorination reactions may occur at elevated temperatures. To advance our understanding of the temperature limits of the reductive dechlorination process and its application to combined remedy approaches (i.e., thermal treatment coupled with bioremediation), investigations targeting microbes capable of chlorinated ethene reductive dechlorination to ethene at elevated temperatures are warranted.

5.5.2 Effect of Elevated Temperatures on *Dhc* Abundance

Quantitative analyses of biomarker genes are frequently used to assess bioremediation potential and to monitor in situ dechlorination processes (21, 23). Since *Dhc* populations grow at the expense of reductive dechlorination reactions, temporal increases in *Dhc* biomarker genes are useful indicators of activity (17). A criticism of using DNA-based analysis to infer activity is that the measurements cannot distinguish active cells from inactive or dead cells. The results of this study demonstrate that the abundances of biomarker genes decreased in cultures incubated at inhibitory temperatures and that quantitative assessments of biomarker genes generally reflected the observed

dechlorination activities. These observations suggest that some *Dhc* cells which were inactive due to elevated temperatures lysed and were not captured in the analysis. For example, the *bvcA* gene, which was initially present at approximately 10^5 gene copies per mL culture fluid, decreased to below the detection limit in cultures in which complete dechlorination to ethene did not occur. The results of this study support the use of *Dhc* biomarker gene abundances for monitoring dechlorination activity at sites undergoing thermal treatment.

5.5.3 Effect of Elevated Temperatures on *Dhc* Gene Expression

The up-regulation of *Dhc* RDase gene expression in cultures incubated at elevated temperatures that prevented reductive dechlorination demonstrated that *Dhc* cells remained active and viable. The survival of *Dhc* cells incubated at elevated temperatures was further supported by the observed recovery of dechlorination activity. Increased RDase gene expression at elevated temperatures in the absence of measurable reductive dechlorination activity suggests that this up-regulation is a general stress response to heat.

The detection of *Dhc* RDase gene transcripts has also been observed in other dechlorination- or growth-inhibited *Dhc* cultures (24, 47). In oxygen-inhibited *Dhc* cultures, Amos et al. (2008) detected *Dhc* RDase gene transcript-per-cell ratios that were, in some cases, similar to or even greater than those in uninhibited cultures (24). The decoupling of dechlorination with net *Dhc* growth has been observed repeatedly (13, 48), and during active dechlorination Johnson et al. (2008) reported the cessation of net growth of *Dhc ethenogenes* strain 195 (47). During the transition of strain 195 to stationary phase, six RDase genes were up-regulated. These findings further support the

suggestion that RDase genes are up-regulated in response to stress such as elevated temperatures, starvation conditions, and the transition from exponential growth to the stationary phase. Because the only identified route for energy generation in *Dhc* is via reductive dechlorination, RDases are vital components of *Dhc* energy metabolism (8). The up-regulation of energy metabolism genes in response to stressors is not unprecedented. For example, during exposure to elevated temperatures, TCA cycle genes were up-regulated in *Escherichia coli* strain MG1655 and in *Staphylococcus aureus* strain ISP794 cultures (49-50).

RDase gene expression has been proposed to be a metabolic indicator that correlates with *Dhc* dechlorination activity (13, 18, 20, 25-28); however, the results of the experiments presented here suggest that RDase gene expression may not correlate with dechlorination activity and that up-regulation of RDase genes expression is a stress response in *Dhc*. Specifically, the analysis of *Dhc* transcripts may lead to erroneous conclusions at sites undergoing thermal treatment and, in general, *Dhc* gene expression measurements should be interpreted cautiously.

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CHAPTER 6

THE EFFECT OF *DEHALOCOCCOIDES* REDUCTIVE DEHALOGENASE GENE AND CULTURE COMPOSITION ON STABLE CARBON ISOTOPE FRACTIONATION OF CHLORINATED ETHENES

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6.1 Abstract

Compound specific stable isotope fractionation (CSIA) has successfully been used to demonstrate reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) in situ; however, at some sites, dechlorination intermediates (i.e., dichloroethenes (DCEs) and vinyl chloride (VC)) accumulate. In order to estimate the biodegradation of dechlorination intermediates, accurate bulk enrichment (ε_{bulk}) factors must be known. Carbon isotope ε_{bulk} factors for DCE and VC dechlorination have been determined in selected cultures, but little is known about the reproducibility of ε_{bulk} factors between independent experiments or how ε_{bulk} factors vary with the *Dhc* strain or reductive dehalogenase (RDase) catalyzing the reaction. The results of this study demonstrate that i) isotope effects are generally reproducible in independent experiments, ii) similar ε_{bulk} factors are produced by *Dhc* strains regardless of the RDase catalyzing the reaction, and iii) fractionation of *cis*-1,2-DCE (*cis*-DCE) and VC in mixed cultures is similar to that measured in pure cultures. Biodegradation extents were calculated based

on the complete range of known ε_{bulk} factors and the maximum biodegradation extent uncertainty was 27%, much lower than the uncertainties for tetrachloroethene (PCE) or trichloroethene (TCE) dechlorination. Therefore, CSIA may yield accurate assessments of biodegradation extent at sites with *cis*-DCE or VC accumulation.

6.2 Introduction

Chlorinated solvents such as tetrachloroethene (PCE) and trichloroethene (TCE) are widespread groundwater contaminants due to extensive use and inappropriate disposal practices (1). Bioremediation represents one approach for the clean-up of chlorinated ethene-contaminated sites (2-4). Anaerobic bioremediation of chlorinated ethenes relies on stepwise reductive dechlorination wherein one carbon-chlorine bond is cleaved and the chlorine atom is replaced with a hydrogen atom (5). In this manner, PCE is dechlorinated to TCE, TCE to a dichloroethene (DCE) (usually *cis*-1,2-DCE (*cis*-DCE)), DCE is dechlorinated to vinyl chloride (VC), and VC is dechlorinated to non-toxic ethene (5). Phylogenetically diverse bacteria are capable of PCE and TCE dechlorination to DCE, but only populations within the *Dehalococcoides* (*Dhc*) genus are capable of dechlorination of DCEs and VC (6). Because *Dhc* are not present at all contaminated sites (5), PCE and TCE may be dechlorinated only to DCEs, causing DCEs to accumulate (7-8). Furthermore, not all *Dhc* strains are capable of respiratory VC dechlorination (9-10) and VC dechlorination is more sensitive to environmental stressors (e.g., elevated temperatures and oxygen exposure) than *cis*-DCE dechlorination (11-12). Hence, VC accumulation may also occur (7, 13).

Processes such as dilution, sorption, and volatilization may contribute to decreasing contaminant concentrations (14-15) and therefore, multiple lines of evidence are required to demonstrate that in situ dechlorination is occurring (16). Compound specific stable isotope analysis (CSIA) relies on the faster rate of biotransformation of light, ^{12}C-containing compounds than heavy, ^{13}C containing compounds (15) and has successfully been used to demonstrate the in situ dechlorination of chlorinated ethenes (17-22). Using CSIA, carbon isotope bulk enrichment (ε_{bulk}) factors are calculated based on the Rayleigh equation (23). At contaminated sites, the in situ biodegradation extent (the fraction of the disappearance of a compound that is due to biodegradation) can be estimated if the shift in the isotope composition and the ε_{bulk} factor are known.

The isotope effects for PCE and TCE dechlorination are highly variable (14). During dechlorination of PCE by *Desulfuromonas michiganensis* and *Geobacter lovleyi* strain SZ, no significant fractionation occurs; therefore, the ε_{bulk} factors for PCE dechlorination by both isolates are smaller in magnitude than -0.4‰ (14). Conversely, the PCE ε_{bulk} factor for dechlorination by *Desulfitobacterium* strain Viet1 is -16.7‰, (14). Similarly, ε_{bulk} factors for dechlorination of TCE range from -2.5 to -18.9‰ (14, 24). Because biodegradation extents are estimated based on ε_{bulk} factors, the larger the range of ε_{bulk} factors, the larger the range of possible biodegradation extents. The biodegradation extent uncertainty is calculated by subtracting the minimum calculated biodegradation extent from the maximum calculated biodegradation extent for a specific shift in isotope composition. The large variability of ε_{bulk} factors for PCE and TCE dechlorination causes the biodegradation extent uncertainty to be up to 89% (14); therefore, accurately estimating the in situ biodegradation extent of PCE and TCE is

challenging (14, 25). Estimating the biodegradation extent of dechlorination intermediates (i.e., DCE and VC) is complex when intermediates are simultaneous being both formed and degraded (26-27). However, if dechlorination proceeds such that a dechlorination intermediate (e.g., *cis*-DCE or VC) has accumulated significantly and is no longer being formed, accurate estimates of the biodegradation extent of the intermediate may be feasible.

Previously determined ε_{bulk} factors for *cis*-DCE and VC dechlorination range from -14.1 to -29.7‰ and from -21.5 to -26.6‰, respectively (24-25). This variability may be due to *Dhc* strain- or enzyme-specific differences. Two reductive dehalogenases (RDases) that catalyze chlorinated ethene dechlorination reactions have been identified, TceA, which catalyzes dechlorination of TCE and *cis*-DCE (28-29) and VcrA, which catalyzes the dechlorination of *cis*-DCE and VC (30). TceA and VcrA were purified from cultures of *Dhc* strain 195 and *Dhc* strain VS, respectively, but *Dhc* strain FL2 possess the *tceA* gene (9) and *Dhc* strain GT possess the *vcrA* gene (31) as well. Based on genetic studies, the *bvcA* gene has been described to catalyzing VC dechlorination in strain BAV1 (32). Studies conducted with *Dhc* pure cultures have only investigated fractionation in cultures containing the *bvcA* and *tceA* genes (25) and only one study has examined the reproducibility of results between independent experiments (14). No studies have determined whether ε_{bulk} factors are consistent between unique strains that catalyze reactions with the same RDase or whether ε_{bulk} factors in mixed cultures are correlated with the presence of a specific *Dhc* strain or RDase. If ε_{bulk} factors are *Dhc* strain- or RDase-specific, it may be possible to predict in situ ε_{bulk} factors based on the organisms present at a contaminated site. The goals of this study were to determine

whether i) isotope effects determined in independent experiments for reactions catalyzed by pure cultures are reproducible, ii) reactions catalyzed by the same RDase present in unique *Dhc* strains are consistent, and iii) isotope effects can be correlated between pure and mixed cultures.

6.3 Materials and Methods

6.3.1 Cultures and Medium Preparation

The following pure *Dhc* cultures were used in this study: strain BAV1 (6), strain FL2 (9), and strain GT (31). Strain BAV1 is capable of respiratory dechlorination of all DCEs and VC (6), strain FL2 is capable of metabolic dechlorination of TCE, *cis*-DCE, and *trans*-1,2-DCE (*trans*-DCE) (9), and strain GT is capable of metabolic dechlorination of TCE, 1,1- and *cis*-DCE, and VC (31). A highly enriched culture containing *Dhc* strain VS was also used (33). Strain VS has the same metabolic substrate range as strain GT ((30, 33); personal communication A. Spormann). Isotope fractionation was also measured in the mixed, PCE-to-ethene dechlorinating Bio-Dechlor INOCULUM (BDI) consortium, which contains strain BAV1, strain FL2, and strain GT (11).

Duplicate or triplicate cultures were inoculated (3% vol/vol) into anaerobic, bicarbonate buffered (30 mM) mineral salts medium (34) amended with vitamins (35) and 1.4 mM Ti(III) citrate. TCE and DCEs were amended to cultures dissolved in 0.1 ml methanol and VC was amended to cultures in gaseous form. Final aqueous concentrations were between 12 and 59 mg/L. *Dhc* pure cultures and strain VS-containing cultures were additionally amended with 4 mL hydrogen and 5 mM acetate.

BDI cultures were amended with only 5 mM lactate. Cultures were incubated at 25ºC in 160-ml serum bottles containing approximately 100 ml of medium.

6.3.2 Analytical Techniques and Sample Collection

Chlorinated ethene and ethene concentrations and isotope compositions were quantified as described previously (34). Briefly, to determine concentrations, headspace or aqueous samples were collected and measured using a gas chromatograph (GC) (34). To determine isotope compositions, aqueous samples (7 mL) were collected and placed in 10 mL vials with 1 mL of 1 M NaOH. Immediately after sample addition, vials were closed with Teflon-lined butyl rubber septa. Headspace samples (0.05 to 1.0 mL) were removed from sample vials incubated at 25 to 60°C and were injected into a GC combustion isotope ratio mass spectrometer as described previously (14, 34). Breifly, the temperature of the oxidation oven was set at 980°C and samples were injected into a gas chromatograph using a split/splitless injector at 250°C. The isotope compositions of samples were calibrated using reference CO_2 gas. Each sample vial was analyzed a minimum of two times.

6.3.3 Isotope Fractionation Calculations

Stable carbon isotope ε_{bulk} factors for dechlorination reactions were calculated according to the Rayleigh model (15)

$$(\varepsilon_{bulk}/1000)\ln(C_t/C_0) = \ln(R_t/R_0) \quad (6.1)$$

where C_t is the number of moles per bottle of the parent compound at time t and C_0 is the initial number of moles of the parent compound per bottle. The R_t/R_0 term was calculated according to the following equation (15)

$$R_t/R_0 = ((1000+ \delta^{13}C_0 + \Delta\delta^{13}C)/(1000+ \delta^{13}C_0)) \quad (6.2)$$

where $\delta^{13}C_0$ is the carbon isotope composition of the parent compound at time zero and $\Delta\delta^{13}C$ is the shift in the carbon isotope composition from time zero to time t.

Due to the removal of samples, decreases in the number of moles of the parent compound were not entirely due to biodegradation. Therefore, in order to correct ε_{bulk} factors for the effect of sample removal, C_t was corrected as follows

$$C_{t,correct} = C_t + C_{r,t-1} \quad (6.3)$$

where $C_{t,correct}$ is the corrected number of moles of the parent compound present per bottle at time t and $C_{r,t-1}$ is the cumulative number of moles of parent compound removed per bottle due to sample collection at time t-1. Similarly, C_0 was also corrected as follows

$$C_{0,correct} = C_0 - C_{r,t-1} \quad (6.4)$$

where $C_{0,correct}$ is the corrected number of moles of the parent compound per bottle that were available for dechlorination at time t. By replacing C_t with $C_{t,correct}$ and C_0 with $C_{0,correct}$ in the Rayleigh model, calculated ε_{bulk} factors only rely upon the number of moles per bottle of the parent compound that were removed due to biodegradation. As described previously (34), to determine ε_{bulk} factors, Rayleigh plots were constructed with $\ln(C_{t,correct}/C_{0,correct})$ plotted versus $\ln(R_t/R_0)$. The slope of the line corresponds to $\varepsilon_{bulk}/1000$. Errors associated with ε_{bulk} factors were determined based on the error of the slope.

To estimate the biodegradation extent of the parent compound based on a known ε_{bulk} factor, the following equation was used (14, 23)

$$B = 1\text{-}e\text{^}((\varepsilon_{bulk}/1000)(ln((1000+\Delta\delta^{13}C)/1000))) \quad (6.5)$$

where B is the biodegradation extent and was calculated for varying shifts in isotope composition ($\Delta\delta^{13}C$) based on known ε_{bulk} factors. In equation 6.5, it is assumed that the initial isotope composition of the parent compound, $\delta^{13}C_0$, is zero.

6.4 Results and Discussion

6.4.1 Reproducibility of ε_{bulk} Factors in Pure Cultures

Carbon ε_{bulk} factors are assumed to be consistent for the dechlorination of a specific compound by a particular bacterial strain. Because the biodegradation extent is based on the ε_{bulk} factor, if ε_{bulk} factors are consistent, biodegradation extent can be estimated accurately as long as the bacterial strain catalyzing the reaction is known. For the reductive dechlorination of chlorinated ethenes, the reproducibility of ε_{bulk} factors between independent experiments has been investigated in only a single case. Carbon ε_{bulk} factors for the fractionation of TCE by *Dhc* strain 195 determined in two independent studies (14, 25) were -9.6 ± 0.4‰ and -13.7 ± 1.8‰ (Table 6.1), a difference of 4.1‰, or 35.2%.

In the current experiment, the fractionation of chlorinated ethenes during dechlorination by strain BAV1 was investigated in order to compare ε_{bulk} factors to those previously reported by Lee et al. (25). The corrected ε_{bulk} factors for *trans*-DCE and VC

dechlorination determined in the current study and by Lee et al. are within standard errors (Figure 6.1, Table 6.1). Carbon isotope ε_{bulk} factors for *cis*-DCE fractionation ranged from -14.9 ± 0.5‰ to -16.9 ± 1.4‰ (Table 6.1), demonstrating a difference in magnitude of only 2.0‰, or 12.6%. For 1,1-DCE dechlorination, ε_{bulk} factors differed by a magnitude of 3.3‰, but because ε_{bulk} factors were low (from -5.1 ± 0.3‰ to -8.4 ± 0.3‰, Table 6.1), this comprised a difference of 48.9%.

The BAV1 cultures used in the current study were maintained independently from those used in the study performed by Lee et al. (25) for over five years. Furthermore, BAV1 cultures were grown in distinct media and isotope compositions were determined at different facilities in these two studies. Yet, the average percentage difference between ε_{bulk} factors was only 16.9% and ε_{bulk} factors differed by a maximum of 3.3‰. These results corroborate the finding that ε_{bulk} factors are not identically reproducible, but that isotope effects are generally consistent for dechlorination reactions catalyzed by pure cultures.

6.4.2 The Effect of RDase Gene on ε_{bulk} Factors

Isotope fractionation is caused by the cleavage of chemical bonds, but the fractionation extent (and ε_{bulk} factor magnitude) are also affected by slow, non-fractionating steps preceding bond cleavage such as binding of the enzyme to the substrate or transport of the substrate across the cell membrane (15). Therefore, it is expected that the isotope effects for reactions catalyzed by the same enzyme in closely related organisms would be similar. In fact, in a previous study, the ε_{bulk} factors for the dechlorination of PCE and TCE catalyzed by the PceA enzyme in *Sulfurospirillum*

multivorans and *Sulfurospirillum halorespirans* were found to be statistically identical (38). Because *Dhc* strains are closely related and some unique strains catalyze reactions with the same RDase, in this study, it was hypothesized that ε_{bulk} factors may be consistent for reactions catalyzed with the same RDase. If this is the case, ε_{bulk} factors could be determined simply by identifying the RDase catalyzing the reaction.

Table 6.1 Compound specific ε_{bulk} factors for the reductive dechlorination of chlorinated ethenes by cultures containing *Dhc*. All values have been corrected for the effects of sample removal.

Culture	TCE		1,1-DCE		*cis*-DCE		*trans*-DCE		Vinyl Chloride	
	ε_{bulk} (‰)	R^2	ε_{bulk} (‰)	R^2	ε_{bulk} (‰)	R^2	ε_{bulk} (‰)	R^2	ε_{bulk} (‰)	R^2
Pure and Highly Enriched Dhc Cultures										
195 (*tceA*)	-13.7 ± 1.8[a] -9.6 ± 0.4[b]	0.95[a] 0.99[b]	NT[a,d] -5.8 ± 0.5[b]	 0.97[b]	NT[a] -21.1 ± 1.8[b]	 0.97[b]	NA[e]		NA	
BAV1 (*bvcA*)	NA		-8.4 ± 0.3[b] -5.1 ± 0.3	0.99[b] 0.97	-16.9 ± 1.4[b] -14.9 ± 0.5	0.98[b] 0.99	-21.4 ± 0.9[b] -20.8 ± 2.8	0.99[b] 0.92	-24.0 ± 2.0[b] -23.2 ± 1.8	0.97[b] 0.96
FL2 (*tceA*)	-8.0 ± 0.4	0.98	NA		-15.8 ± 1.1	0.97	NT		NA	
GT (*vcrA*)	NT		NT		-21.6 ± 1.3	0.97	NA		-23.8 ± 1.1	0.99
VS (*vcrA*)	NT		NT		-17.6 ± 2.7	0.89	NA		-22.1 ± 1.3	0.97
Mixed Cultures										
ANAS[b]	-16.0 ± 0.6	0.99	-23.9 ± 1.2	0.98	-29.7 ± 1.6	0.99	-28.3 ± 1.4	0.99	-22.7 ± 0.8	0.99
BDI	NT		NT		-25.3 ± 1.0	0.99	NT		-18.5 ± 1.5	0.96
KB1[c]	-2.5 to -13.8	0.91	NT		-14.1 to -20.4	0.92	NA		-21.5 to -26.6	0.96

Table 6.1 (continued) Compound specific ε_{bulk} factors for the reductive dechlorination of chlorinated ethenes by cultures containing *Dhc*. Data collected in the current study that is shown in bold has been corrected for the effects of sample removal.

[a] Data is from Chichocka et al. (14).
[b] Data is from Lee et al. (25).
[c] Data is from Bloom et al. (24), Duhamel et al. (36), and Slater et al. (37).
[d] NT indicates that fractionation was not quantified although the chlorinated ethene supports metabolic growth.
[e] NA indicates that fractionation was not quantified because the chlorinated ethene does not serve as a metabolic electron acceptor.

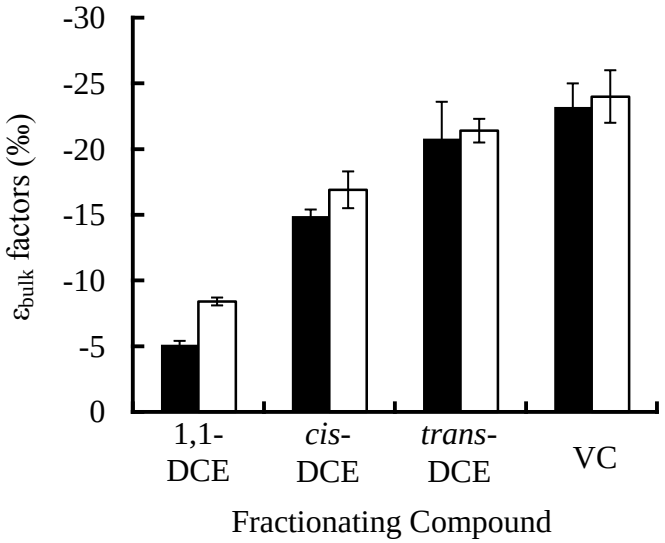

Figure 6.1 Comparison of corrected ε_{bulk} factors for dechlorination of 1,1-, *cis*-, and *trans*-DCE and VC by strain BAV1 as determined in the current study (black bars) and as reported previously (white bars; (25)). Error bars represent one standard deviation.

Dhc strain 195 and strain FL2 both possess the *tceA* gene which encodes for TceA, an enzyme that catalyzes the dechlorination of TCE and *cis*-DCE (9, 29). In the case of both TCE and *cis*-DCE, larger magnitude ε_{bulk} factors were measured for dechlorination reactions catalyzed by strain 195 (Figure 6.2). The average ε_{bulk} factor from two independent experiments (14, 25) for the dechlorination of TCE by strain 195, was 11.7 ± 2.9‰, a difference of 3.7‰ as compared to the dechlorination of TCE by strain FL2 (Figure 6.2). The difference between the ε_{bulk} factors measured for the dechlorination of TCE by strain 195 in the independent experiments, however, was 4.1‰ (Table 6.1). The difference between the ε_{bulk} factors for TCE dechlorination by strain FL2 as measured in the current study and by strain 195 as measured in the experiment performed by Lee et al. (25) was only 1.6‰. Therefore, the difference in ε_{bulk} factors measured for the same *Dhc* strain in independent experiments was greater than the difference measured between *Dhc* strains. For the dechlorination of *cis*-DCE, ε_{bulk} factors for strain 195 and strain FL2 differed by 5.3‰ or 28.7%. This variation is within that reported for pure cultures analyzed in independent studies. Interestingly, the ε_{bulk} factor for *cis*-DCE dechlorination by strain FL2 was statistically identical to that for BAV1 (Figure 6.2), which does not contain the *tceA* gene (32). Similarly, the ε_{bulk} factor for dechlorination of *cis*-DCE by strain 195 was statistically identical to that for strain GT (Figure 6.2), which does not contain the *tceA* gene and likely catalyzes *cis*-DCE dechlorination with VcrA (31).

Dhc Strain GT and strain VS both possess the *vcrA* gene which encodes for VcrA, an enzyme that catalyzes the dechlorination of *cis*-DCE and VC (30-31). Both ε_{bulk} factors for *cis*-DCE and VC are higher in magnitude for dechlorination reactions

catalyzed by strain GT (Figure 6.2), but ε_{bulk} factors for identical dechlorination reactions are within statistical error (Table 6.1). Interestingly, for the dechlorination of *cis*-DCE, the ε_{bulk} factor of strain GT is most similar to that of strain 195 whereas the ε_{bulk} factor of strain VS is most similar to that of strain FL2 (Figure 6.2). Similarly, the ε_{bulk} factor for the dechlorination of VC by strain GT is most similar to that for strain BAV1 (Figure 6.3).

Carbon ε_{bulk} factors for chlorinated ethene dechlorination reactions catalyzed by the same strain are not identical between independent experiments and, therefore, it was not expected that reactions catalyzed by the same enzyme in unique strains would be identical. It was, however, expected that ε_{bulk} factors would be more similar for reactions catalyzed by the same enzyme than for reactions catalyzed by unique enzymes. Unexpectedly, most ε_{bulk} factors were more similar between *Dhc* isolates catalyzing reactions with different RDases than for *Dhc* isolates catalyzing reactions with the same RDase (Figure 6.2).

All of the percentage differences between ε_{bulk} factors for reactions catalyzed by the same enzyme in distinct *Dhc* strains were within the variability reported between ε_{bulk} factors determined in independent experiments for the same strain. However, this is also true for all of the dechlorination reactions catalyzed by pure and highly enriched *Dhc* cultures for which ε_{bulk} factors were determined. For example, ε_{bulk} factors for dechlorination of *cis*-DCE ranged from -14.9 to -21.6, but this corresponds to a percentage difference of only 36.7%, less than the differences measured for the dechlorination of 1,1-DCE by strain BAV1 in two independent experiments. Therefore,

in the case of all of the *Dhc* strains that were analyzed, isotope effects are similar regardless of the specific RDase or strain catalyzing the reaction.

6.4.3 Consistency of ε_{bulk} Factors Between Pure and Mixed Cultures

Carbon ε_{bulk} factors are based on the relative rates of cleavage of ^{13}C versus ^{12}C bonds (15) and therefore, the presence of bacterial strains that do not degrade or impact the distribution of substrates should not affect ε_{bulk} factors. Hence, ε_{bulk} factors determined in pure and mixed cultures should be similar as long as the reaction of interest is catalyzed by the same organism in both cultures. In this study, it was hypothesized that ε_{bulk} factors for reactions catalyzed by known populations in mixed cultures could be correlated to the ε_{bulk} factors measured for reactions catalyzed by the same populations in pure culture. For example, *Dhc* strain BAV1 and strain GT are the only organisms in the BDI consortium capable of metabolic VC dechlorination (39). The ε_{bulk} factor for VC dechlorination by these strains was determined in pure cultures (Table 6.1) and it was expected that the ε_{bulk} factor for VC dechlorination in the BDI consortium would be similar to these values. Similarly, *Dhc* strain BAV1, strain FL2, and strain GT are the only organisms capable of metabolic *cis*-DCE dechlorination in the BDI consortium (39) and hence the ε_{bulk} factor for *cis*-DCE dechlorination in the BDI consortium was expected to be similar to these values.

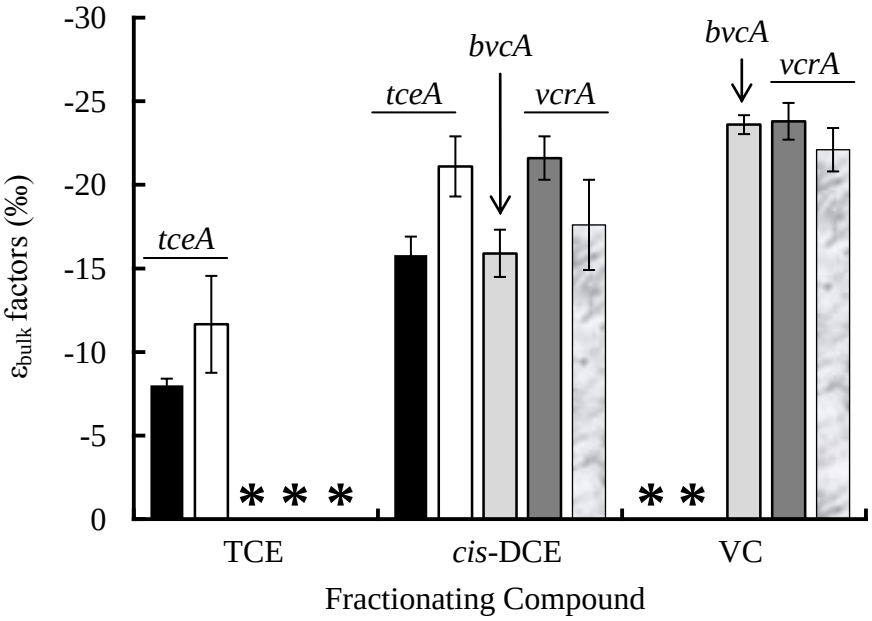

Figure 6.2 Comparison of ε_{bulk} factors for dechlorination of TCE, *cis*-DCE, and VC by strain FL2 (black bars), strain 195 (white bars; values are the average of the values reported by Cichocka et al. (14) and Lee et al. (25)), strain BAV1 (gray bars; values are the average of corrected values from this study and values reported by Lee et al. (25)), strain GT (dark gray bars), and strain VS (marbled bars). All shown ε_{bulk} factors from the current study were corrected for the effects of sample removal. Error bars represent one standard deviation. Asteriks indicate that ε_{bulk} factors are not available.

In the BDI cultures amended with VC, the the ε_{bulk} factor was 5.5‰ less than the largest value measured in pure cultures of *Dhc* strain BAV1 and strain GT (Table 6.1). Due to the large magnitude of VC ε_{bulk} factors, this difference correlates to a percentage difference of only 25.9%, variability that is within that reported between ε_{bulk} factors determined in independent experiments for the same strain. The ε_{bulk} factor for dechlorination of *cis*-DCE in the BDI consortium was 10.4‰ greater than the ε_{bulk} factor measured in strain BAV1 pure cultures, the smallest ε_{bulk} factor measured in *Dhc* pure cultures (Table 6.1). The difference between these ε_{bulk} factors is 51.7%, slightly above the percentage difference measured in independent experiments for the same *Dhc* strain.

Previous studies have also investigated isotope fractionation of *cis*-DCE and VC in mixed cultures. Pure cultures of the *Dhc* strains catalyzing *cis*-DCE and VC dechlorination have not been isolated and therefore, ε_{bulk} factors have not been determined for the specific *Dhc* strains catalyzing the reactions. Because ε_{bulk} factors are similar for all measured *Dhc* strains, it was hypothesized that ε_{bulk} factors in mixed cultures would be similar to ε_{bulk} factors determined in pure *Dhc* cultures. In fact, the ε_{bulk} factor for dechlorination of VC in the mixed ANAS culture is -22.7 ± 0.8‰ (25), precisely within the range of ε_{bulk} factors for VC dechlorination by *Dhc* isolates (Table 6.1). The ε_{bulk} factor for dechlorination of *cis*-DCE in the ANAS culture is -29.7 ± 1.6‰ (25), which is greater in magnitude by 8.1‰, than the value measured in any pure culture (Table 6.1). Yet, the 8.1‰ difference in magnitude correlates only to a difference of 31.6%, which is within the variability measured between pure cultures analyzed in independent studies. Multiple studies have examined isotope fractionation in the KB1 consortium and reported ε_{bulk} factors for *cis*-DCE and VC dechlorination have ranged

from -14.1 to -20.4‰ and from -21.5 to -26.6‰, respectively (24, 37). The ranges of ε_{bulk} factors determined in the KB1 consortium nearly flank those reported for pure cultures, which range from -14.9 to -21.6‰ and from -22.1 to -24.0‰ for *cis*-DCE and VC dechlorination, respectively (Table 6.1).

Overall, in five out six cases, isotope effects measured in mixed cultures for the dechlorination of *cis*-DCE and VC were within the variability of that reported for ε_{bulk} factors measured in pure cultures in independent studies. These results demonstrate that isotope effects are consistent between *Dhc* pure and mixed cultures. Furthermore, the ANAS and KB1 cultures contain *Dhc* strains for which ε_{bulk} factors have not yet been determined, but, even in the cases of the ANAS and KB1 cultures, ε_{bulk} factors determined in mixed cultures are similar to values determined in other *Dhc* pure cultures. These results support the hypothesis that isotope effects for dechlorination reactions catalyzed by *Dhc* are similar regardless of the specific strain or RDase catalyzing the reaction.

6.4.4 Implications for Estimates of Biodegradation Extent

In situ biodegradation extent is calculated according to the Rayleigh model based on the shift in isotope composition and the value of the ε_{bulk} factor. To assess the impact of the variability of ε_{bulk} factors on the in situ quantification of *cis*-DCE and VC dechlorination, biodegradation extents were determined based on both the minimum and maximum ε_{bulk} factors listed in Table 6.1 for shifts in isotope composition from 0‰ to 80‰ (Figure 6.3). For *cis*-DCE dechlorination, ε_{bulk} factors varied from -14.1 to -29.7‰ (Table 6.1). The calculated biodegradation extent based on an isotope shift of 10‰ and an ε_{bulk} factor of -14.9‰ was 51.6%. Conversely, also assuming an isotope shift of 10‰,

the calculated biodegradation extent based on the largest magnitude ε_{bulk} factor (i.e, -29.7‰) was 29.2% (Figure 6.3). Therefore, for *cis*-DCE dechlorination, the calculated uncertainty for an isotope shift of 10‰ is 22.5%. Assuming a shift of 60‰, biodegradation extents ranged from 86.7 to 98.6% and therefore the uncertainty is 11.9%. The maximum *cis*-DCE biodegradation extent uncertainty is 26.8%, for a shift in isotope composition of 20‰. Carbon isotope ε_{bulk} factors for VC dechlorination only varied over 8.1‰, from -18.5 to -26.6 (Table 6.1), and therefore, the maximum uncertainty was only 13.3% for a shift in isotope composition of 22‰ (Figure 6.3).

Although ε_{bulk} factors for dechlorination of *cis*-DCE and VC varied by as much as 15.6‰, ε_{bulk} factors were all the same order of magnitude and significant fractionation occurred in all cases. Conversely, no measurable fractionation occurs during PCE dechlorination by *Desulfuromonas michiganensis* and *Geobacter lovleyi* strain SZ (14). In pure cultures, measured ε_{bulk} factors for the dechlorination of PCE range over 2 orders-of-magnitude, from 0.5 ± 0.2 to 16.7 ± 4.5‰ (14). Similarly, for TCE dechlorination, measured ε_{bulk} factors range from 3.5 ± 0.2 to 18.9 ± 1.0‰ (14). Fractionation of *cis*-DCE and VC is less variable likely because *cis*-DCE and VC dechlorination reactions are only catalyzed by *Dhc* rather than by the phylogenetically diverse bacteria that catalyze PCE and TCE dechlorination (5, 14). Because of the large range of ε_{bulk} factors for PCE and TCE dechlorination, the maximum uncertainties associated with the biodegradation extents of PCE and TCE dechlorination are 89 and 55%, much larger than those for *cis*-DCE and VC dechlorination (14). The small range of ε_{bulk} factors for *cis*-DCE and VC dechlorination makes CSIA a promising approach for the quantification of biodegradation extent at sites with *cis*-DCE or VC accumulations.

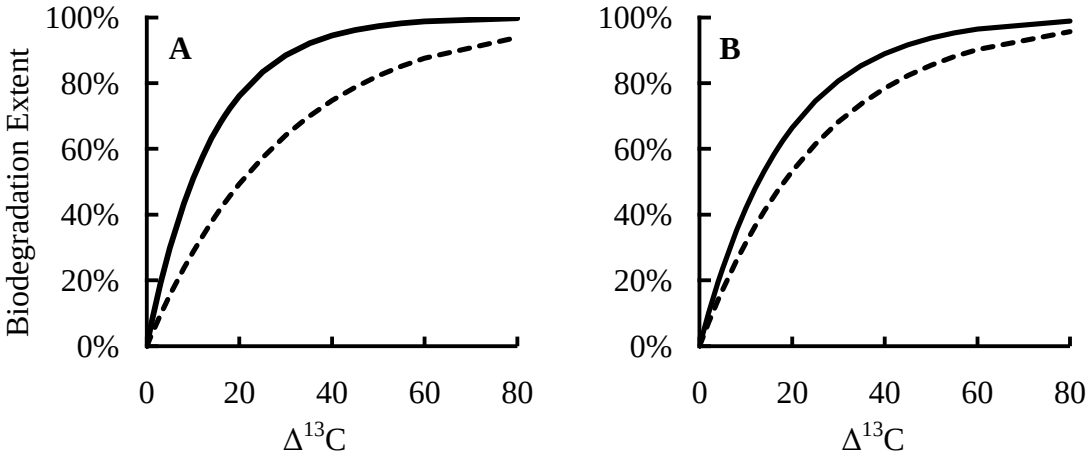

Figure 6.3 Calculated biodegradation extent of *cis*-DCE (A) and of VC (B) versus shifts in isotope composition based on the range of corrected ε_{bulk} factors shown in Table 6.1. Solid lines are estimates based on minimum ε_{bulk} factors and dashed lines are based on maximum ε_{bulk} factors.

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CHAPTER 7

STABLE CARBON ISOTOPE FRACTIONATION OF 1,2-DICHLOROPROPANE DURING DICHLOROELIMINATION BY *DEHALOCOCCOIDES* POPULATIONS

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7.1 Abstract

The isotope fractionation of 1,2-dichloropropane (1,2-D) during dichloroelimination to propene by *Dehalococcoides* populations was explored in laboratory experiments in order to provide data for the characterization of the fate of 1,2-D in heterogeneous subsurface systems. Compound specific stable carbon isotope analysis (CSIA) was used to determine the bulk enrichment factors (ε_{bulk}), reactive position specific enrichment factors ($\varepsilon_{reactive}$), and apparent kinetic isotope effect (AKIE) values for 1,2-D dichloroelimination in two distinct *Dehalococcoides*-containing cultures. The ε_{bulk} factors calculated in the two cultures were statistically identical, -10.8 ± 0.9 and -11.3 ± 0.8‰, even though the cultures were derived from geographically distinct locations. AKIE values for 1,2-D dichloroelimination assuming stepwise and concerted reaction mechanisms were approximately 1.033 and 1.017, respectively. These values are within the range of previously reported values for dichloroelimination reactions and

were equivalent to values reported for biotic 1,2-dichloroethane and abiotic 1,1,2,2,-tetrachloroethane and pentachloroethane dichloroelimination reactions.

7.2 Introduction

1,2-Dichloropropane (1,2-D) has been used extensively as a soil fumigant in agriculture and is generated during the production of propylene oxide and chlorinated compounds, including tetrachloroethene and carbon tetrachloride (1-4). 1,2-D is a potential carcinogen and therefore, is regulated by the U.S. Environmental Protection Agency at a maximum contaminant level in drinking water of 5 ppb (5). Because 1,2-D is a contaminant of concern at over 100 U.S. Superfund sites (6), tools to assess the fate of 1,2-D in subsurface environments are needed.

Microcosm and field studies have demonstrated that 1,2-D is recalcitrant under aerobic and nitrate-reducing conditions, but degraded under anaerobic conditions (7). BL-DC-8 and BL-DC-9, isolates comprising a novel genus within the Chloroflexi phylum (8), *Dehalobacter* populations (9), *Dehalococcoides* populations (10), and *Desulfitobacterium dichloroeliminans* strain DCA1 (11) can transform 1,2-D to propene under anaerobic conditions. Strains BL-DC-8, BL-DC-9, DCA1 and *Dehalococcoides* populations convert 1,2-D to propene via a dichloroelimination reaction without the formation of intermediates (3, 8, 11). In subsurface environments, however, physical processes such as sorption, volatilization, and dilution also affect the concentration and fate of 1,2-D.

In general, quantitatively differentiating the effects of biotransformations from physical processes on contaminants in heterogeneous subsurface systems is challenging (12). Compound specific stable carbon isotope analysis (CSIA) is used to quantify the isotope enrichment of compounds undergoing biodegradation and has been applied to assess the *in situ* biodegradation of chlorinated ethenes (13-18) and chlorinated ethanes (13, 14). To apply CSIA for quantifying *in situ* contaminant degradation using the Rayleigh equation, the carbon isotope enrichment factor (ε_{bulk}) is used as a parameter (19). The ε_{bulk} factor is derived from defined laboratory experiments, where the only sink of the contaminant is, for example, biodegradation. Laboratory-derived ε_{bulk} factors can also be converted to reactive position specific enrichment factors ($\varepsilon_{reactive}$) and apparent kinetic isotope effect (AKIE) values to obtain information about the biochemical transformation mechanism. Because AKIE values are corrected for the isotope composition of atoms that are not at the reactive site and for the effects of intramolecular competition, AKIE values describe the isotope effect associated specifically with the chemical bond cleavage (20). The semi-empirical Streitwieser limits provide reaction-specific maximum values for isotope effects which can be used as a simplified theoretical framework for interpretation of AKIE values (21). While position-specific isotope analysis provides even greater information regarding bond cleavage, CSIA was performed in this study because it is applicable to environmental samples.

In this study, ε_{bulk} and $\varepsilon_{reactive}$ factors for 1,2-D dichloroelimination were determined in two highly enriched, *Dehalococcoides*-containing cultures derived from geographically distinct locations (10). This is the first report of isotope enrichment factors for 1,2-D dichloroelimination. Furthermore, using previously reported ε_{bulk}

factors, AKIE values for other dichloroelimination reactions were calculated to serve as a comparison to values for 1,2-D dichloroelimination.

7.3 Experimental Section

7.3.1 Cultures and Medium Preparation

Non-methanogenic RC and KS enrichment cultures were derived from sediments from the Red Cedar River in Michigan and the King Salmon River in Alaska, respectively (10). Duplicate cultures were inoculated (3% vol/vol) into anoxic, bicarbonate buffered (30 mM) mineral salts medium (22) amended with vitamins (23), 1.4 mM Ti(III) citrate, 5 mM acetate, 3 to 4 ml gaseous H_2, and 1,2-D dissolved in 0.1 ml methanol. RC and KS cultures were amended with 1.7 and 1.1 µl of 1,2-D, respectively. Cultures were incubated at 25ºC in 160-ml serum bottles containing approximately 100 ml of medium.

7.3.2 Analytical Techniques

To quantify 1,2-D and propene concentrations, headspace or aqueous samples were collected and measured using a gas chromatograph (GC) as described previously (24, 25). For analysis of carbon isotope composition, 7 mL aqueous samples were collected and placed in 10 ml vials with 1 ml of 1 M NaOH to inhibit metabolic activity. Sample vials were sealed with Teflon-lined septa and stored at 4°C. Each sample vial was analyzed a minimum of two times using a GC combustion isotope ratio mass spectrometer and carbon isotope compositions were quantified as described previously

(25), except that sample vials were heated to 60°C prior to the removal of 0.7 ml headspace samples.

7.3.3 Calculations

Stable carbon isotope ε_{bulk} factors for 1,2-D dichloroelimination were calculated according to the Rayleigh model (21, 25)

$$ln((1000+ \delta^{13}C_0 + \Delta\delta^{13}C)/(1000+ \delta^{13}C_0)) = (\varepsilon_{bulk}/1000)ln(f) \quad (7.1)$$

where $\delta^{13}C_0$ is the carbon isotope composition of 1,2-D at time zero, $\Delta\delta^{13}C$ is the change in the carbon isotope composition from time zero to time t, and f is the molar fraction of 1,2-D remaining at time t. Carbon isotope $\varepsilon_{reactive}$ factors, which are corrected for the presence of nonreactive positions, were calculated for both stepwise and concerted reactions according to (21)

$$ln((1000+\delta^{13}C_0 + (n/x)\,\Delta\delta^{13}C)/(1000+\delta^{13}C_0)) = (\varepsilon_{reactive}/1000)ln(f) \quad (7.2)$$

where n is the number of carbon atoms in the molecule (in the case of 1,2-D, n = 3) and x is the number of carbon atoms in the reactive position (in the case of a stepwise reaction, x = 1 and in the case of a concerted reaction, x = 2, assuming identical KIE values). AKIE values for 1,2-D dichloroelimination were calculated according to (21)

$$AKIE = 1/(1+(z * \varepsilon_{reactive}/1000)) \quad (7.3)$$

where z, the number of indistinguishable reactive sites, is a correction for the effects of intramolecular competition (in the case of 1,2-D, z = 1). In order to compare carbon isotope fractionation during various dichloroelimination reactions, AKIE values for other dichloroelimination reactions were approximated from reported ε_{bulk} values according to (21)

$$AKIE \approx 1/(1+z*(n/x)*\varepsilon_{bulk}/1000) \quad (7.4)$$

where compound-specific values for z, n, and x, are shown in Table 7.1.

7.4 Results and Discussion

7.4.1 Enrichment of ^{13}C in 1,2-D During Microbially Catalyzed Dichloroelimination to Propene

In freshly inoculated RC cultures, dichloroelimination to propene began after a lag period of 3 days. More than 90% of 1,2-D was transformed to propene within 6 days after the lag period. Similarly, in KS cultures, more than 90% of 1,2-D was transformed to propene in 11 days after a lag period of approximately 15 days. The 1,2-D transformation rates were 2.57 ± 0.07 and 1.08 ± 0.12 µmoles per day in RC and in KS cultures, respectively. In both cultures, 1,2-D was significantly enriched in ^{13}C during transformation to propene (Figure 7.1). The initial 1,2-D isotope composition in cultures RC and KS differs because 1,2-D was obtained from different sources (Supelco for culture RC; Riedel-de Haën for culture KS). Isotopically depleted propene was formed in both cultures, verifying isotope fractionation.

Table 7.1 Compound specific values used for the calculation of AKIE values.

Fractionating Compound	n	Stepwise		Concerted	
		x	z	x	z
1,2-D	3	1	1	2	1
1,2-DCA	2	2[a]	2	2	1
1,1,2-TCA	2	1	1	2	1
1,1,2,2-TeCA	2	2[a]	2	2	1
pentachloroethane	2	1	1	2	1
hexachloroethane	2	2[a]	2	2	1
γ-HCH	6	2	2	2	1

[a] No correction for nonreactive positions is required in symmetric molecules and therefore the "n/x" term in equation 7.4 is equal to 1 (21).

In both RC and KS cultures, the total amount of volatile organics in the system (i.e., 1,2-D and propene) became depleted in ^{13}C as degradation continued; yielding a poor isotope balance. For example, in KS cultures that had consumed 1,2-D to below the detection limit of 0.5 mg/L, the isotope composition of propene was -27.9 ± 0.3‰, which is significantly depleted in ^{13}C compared to the initial isotope composition of 1,2-D, which was -24.3 ± 0.5‰. The depletion of ^{13}C in the cumulative organic volatiles in the cultures was a result of the preferential removal of 1,2-D during isotope composition sample collection. Because Henry's constants for 1,2-D and propene are 0.12 and 8.54, respectively, approximately 91% (mole/mole) of 1,2-D is in the aqueous phase while only approximately 11% (mole/mole) of propene is in the aqueous phase in these cultures. Only the aqueous phase was sampled for isotope analysis of 1,2-D and propene, and therefore, about 4- to 5-fold more 1,2-D than propene was removed, resulting in the preferential removal of ^{13}C-enriched substrate and leading to a depletion of ^{13}C in cultures. Correcting the isotope balance for the losses due to sample collection, the expected isotope composition in KS cultures following complete transformation of 1,2-D was calculated to be -28.3 ± 1.3‰. This value is statistically identical to the final isotope composition of propene, -27.9 ± 0.3‰, demonstrating a closed isotope balance (Figure 7.1). The depletion of ^{13}C in RC cultures was accounted for in the same manner. The final isotope composition of propene in the RC cultures was lower than the expected isotope composition likely because only 93% of the initial dose of 1,2-D was transformed to propene prior to collection of the final sample (Figure 7.1).

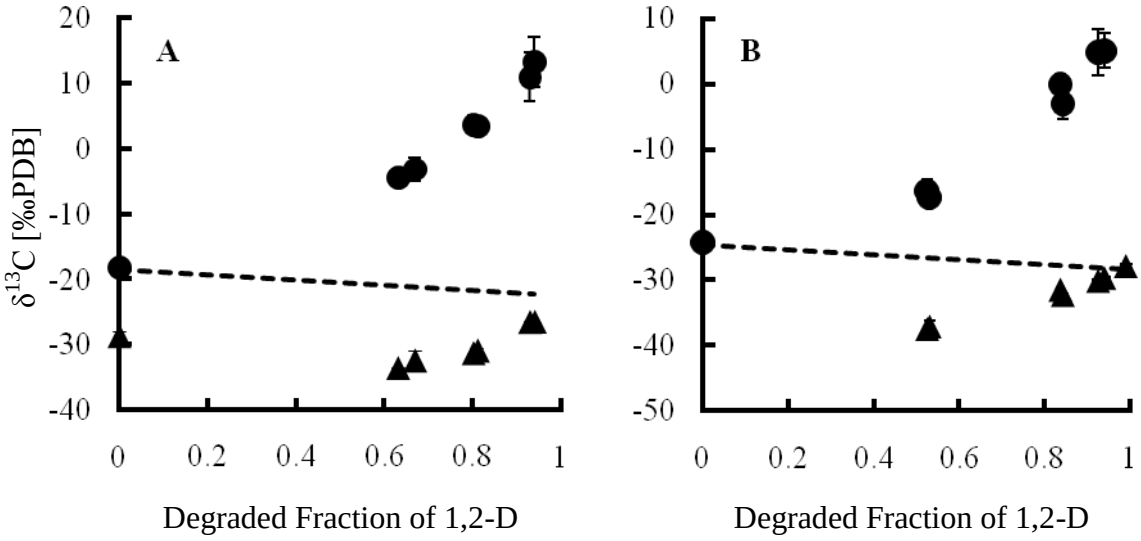

Figure 7.1 The carbon isotope composition of 1,2-D (circles) and propene (triangles) in cultures RC (A) and KS (B) during dichloroelimination. Dashed lines indicate the expected isotope composition of the total volatile organics in the system (i.e., 1,2-D and propene) corrected based on losses due to sample removal. Isotope values are reported in δ-notation relative to the Vienna Pee Dee Belemnite (PDB) standard. Data were averaged from duplicate isotope measurements and error bars depict one standard deviation (1σ).

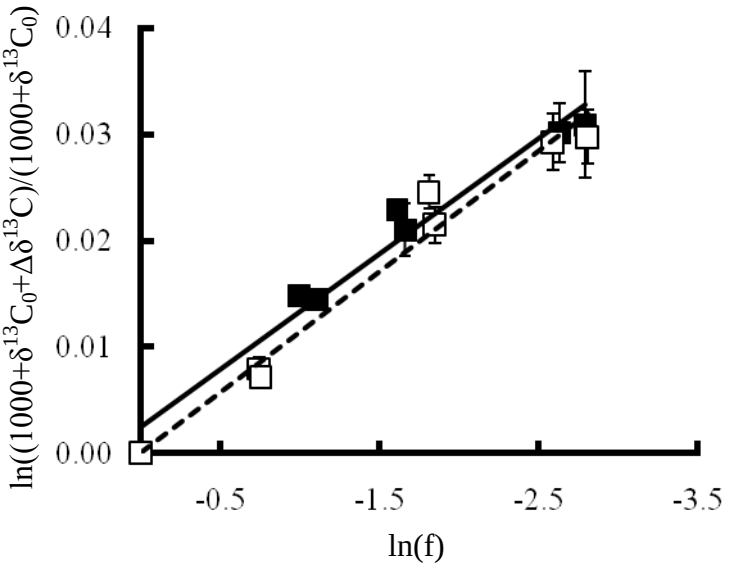

Figure 7.2 Rayleigh plots showing linear regression for 1,2-D transformation experiments in RC (filled squares, solid line) and KS cultures (open squares, dashed line). For both data sets, R^2 values were greater than 0.96. Data were averaged from duplicate isotope measurements and error bars depict one standard deviation (1σ).

7.4.2 Bulk Stable Carbon Isotope Enrichment Factors (ε_{bulk})

In both cultures, the Rayleigh model described isotope fractionation during transformation of 1,2-D to propene (Figure 7.2). Although the enrichment cultures were obtained from geographically distinct locations, harbor unique microbial populations (10), and demonstrate different 1,2-D dichloroelimination rates, the ε_{bulk} factors calculated for RC and KS cultures, -10.8 ± 0.9 and -11.3 ± 0.8‰, respectively, were statistically identical (Table 7.2). These findings suggest that carbon isotope ε_{bulk} factors for 1,2-D transformation by *Dehalococcoides* populations are consistent and therefore may be applied to demonstrate *in situ* natural attenuation of 1,2-D and to assess the extent of 1,2-D biodegradation, although it would be recommendable to corroborate these findings with in further studies.

7.4.3 Apparent Kinetic Isotope Effect (AKIE) Values

AKIE values were calculated to characterize the isotope effect of the cleavage of the chemical bond at the reactive position of the 1,2-D molecule. While two C-Cl bonds are cleaved during the dichloroelimination reaction, it is unknown if the reaction proceeds via a stepwise mode (i.e., one C-Cl bond is broken in the transition state and the rate-limiting step involves only one carbon atom), or via a concerted mode (i.e., two C-Cl bonds are broken simultaneously and the rate-limiting step involves two carbon atoms). Therefore, AKIE values were calculated assuming both stepwise and concerted scenarios.

Assuming that the 1,2-D dichloroelimination reaction is stepwise, involving the cleavage of one C-Cl bond in the transition state, $\varepsilon_{reactive}$ factors are -31.5 ± 2.7 and -32.1 ± 2.5‰ and AKIE values were 1.0325 ± 0.0029 and 1.0332 ± 0.0027 for cultures RC and

KS, respectively. Assuming that the reaction is concerted, involving the simultaneous cleavage of both C-Cl bonds, AKIE values were 1.0164 ± 0.0014 and 1.0167 ± 0.0013 for cultures RC and KS, respectively (Table 7.2). The Streitwieser semiclassical limit for the KIE of the cleavage of a C-Cl bond is 1.057, but measured AKIE values are generally below this value due to the effects of slow, non-fractionating steps preceding bond cleavage, (e.g., binding of the substrate to the enzyme). Furthermore, the Streitwieser limits assume that bonds are completely broken in the transition state of a reaction which is not always the case (21) but these limits allow a broad mechanistic interpretation. Therefore, if AKIE values calculated assuming a stepwise reaction are greater than the Streitwieser limit, the reaction likely proceeds via a concerted mechanism according to a broad classification. In the case of dichloroelimination of 1,2-D, however, the reaction mechanism cannot be absolutely classified because AKIE values calculated assuming both stepwise and concerted reaction mechanisms are significantly below 1.057.

The AKIE values for 1,2-D dichloroelimination were compared to values for dichloroelimination of 1,2-dichloroethane (1,2-DCA), 1,1,2-trichloroethane (1,1,2-TCA), 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA), pentachloroethane, hexachloroethane, and gamma-hexachlorocyclohexane (γ-HCH) (13, 26-30), which were calculated assuming that the effects of secondary KIEs are negligible. The dichloroelimination of 1,2-DCA by two distinct mixed cultures (13), of 1,1,2,2-TeCA by Cu-plated iron (*27*), and of pentachloroethane by Cr(II) (28) demonstrate AKIE values highly similar to those calculated for the *Dehalococcoides*-catalyzed 1,2-D dichloroelimination reaction (Table 7.2). Interestingly, *Dehalococcoides* populations also catalyzed the dichloroelimination of 1,2-DCA in mixed culture D, (also known as KB-1 (31)) and most likely, at least

partially, in culture C (Table 7.2; (32)), which both demonstrated similar AKIE values to those observed for 1,2-D dichloroelimination ((13); personal communication from E. Edwards).

However, reported AKIE values for dichloroelimination reactions vary widely. As shown in Table 7.2, AKIE values calculated assuming a concerted reaction mechanism varied from 1.0020 ± 0.0002 for dichloroelimination of 1,1,2-TCA to 1.0332 ± 0.0011 for dichloroelimination of 1,2-DCA, in both cases, in microcosms constructed from Louisiana soil and groundwater (29). Similarly, calculated AKIE values assuming stepwise reactions ranged from 1.0040 ± 0.0004 to 1.0686 ± 0.0022, again, for the dichloroelimination of 1,1,2-TCA and 1,2-DCA, respectively, in microcosms constructed from Louisiana soil and groundwater (29). Interestingly, in this case, the AKIE value for the dichloroelimination of 1,2-DCA assuming a stepwise reaction is greater than 1.057, the Streitwieser semiclassical limit for the cleavage of a C-Cl bond, indicating that the dichloroelimination of 1,2-DCA in the Louisiana microcosm likely occurs via a concerted reaction mechanism (21). However, carbon isotope fractionation during dichloroelimination of 1,2-DCA has been measured in a number of cultures and calculated AKIE values do not consistently eliminate stepwise reaction mechanisms (*13*). In fact, Hirschorn et al., (2007) (13) reported that calculated AKIE values vary significantly, from 1.0148 ± 0.0004 to 1.0346 ± 0.0010 assuming a stepwise reaction and from 1.0074 ± 0.0002 and 1.0170 ± 0.0005 assuming a concerted reaction, even among enrichment cultures obtained from the same location and amended with the same electron donor (Table 7.2). In this case, because enrichment cultures were maintained separately for several years, differences in carbon isotope fractionation may be due to the presence

of different microbial populations. Interestingly, microbial lindane dichloroelimination, thought to function via a stepwise reaction, also had similar AKIE values (Table 7.2) (26). However, variation in carbon isotope enrichment also occurs in abiotic systems employing the same reactant. For example, Elsner et al., (2007) (27) and Hofstetter et al., (2007) (28) both monitored isotope fractionation of 1,1,2,2-TeCA during dichloroelimination by Cr(II), but AKIE values were inconsistent, 1.0374 ± 0.0012 and 1.0261 ± 0.0012, respectively, assuming stepwise reactions and 1.0184 ± 0.0006 and 1.0129 ± 0.0012, respectively, assuming concerted reactions.

These results demonstrate that AKIE values may vary significantly based on the microbial community or experimental procedures employed and that AKIE values are certainly not consistent for dichloroelimination reactions in general. Therefore, it is particularly remarkable that the ε_{bulk}, and consequently, AKIE values calculated for dichloroelimination of 1,2-D are statistically identical in two distinct enrichment cultures. The consistency of the isotope effects between these cultures indicates that CSIA may be a promising approach to verify and quantify 1,2-D dichloroelimination in subsurface environments.

Table 7.2 Stable carbon isotope ε_{bulk} and calculated AKIE values assuming both stepwise and concerted dichloroelimination reactions.

Fractionating Compound	Reactant	ε_{bulk}	AKIE Value		Reference
			Stepwise	Concerted	
1,2-D	Culture RC	-10.8 ± 0.9‰	1.0325 ± 0.0029	1.0164 ± 0.0014	This study
	Culture KS	-11.3 ± 0.8‰	1.0332 ± 0.0027	1.0167 ± 0.0013	This study
1,2-DCA	Cultures A and B	-7.3 ± 0.2‰	1.0148 ± 0.0004[a]	1.0074 ± 0.0002[a]	(*13*)
	Cultures C and D	-16.7 ± 0.5‰	1.0346 ± 0.0010[a]	1.0170 ± 0.0005[a]	(*13*)
	LA microcosm	-32.1 ± 1.1‰	1.0686 ± 0.0022[a]	1.0332 ± 0.0011[a]	(*29*)
	Zn(0)	-29.7 ± 1.5‰	1.0632 ± 0.0030[a]	1.0306 ± 0.0002[a]	(*30*)
1,1,2-TCA	LA microcosm	-2.0 ± 0.2‰	1.0040 ± 0.0004[a]	1.0020 ± 0.0002[a]	(*29*)
1,1,2,2-TeCA	Cr(II)	-18.0 ± 0.5‰	1.0374 ± 0.0012	1.0184 ± 0.0006	(*27*)
	Cr(II)	-12.7 ± 1.2‰	1.0261 ± 0.0012	1.0129 ± 0.0012[a]	(*28*)
	Cu-plated iron	-17.0 ± 0.6‰	1.0351 ± 0.0012	1.0173 ± 0.0006	(*27*)
	Fe	-19.3 ± 0.7‰	1.0401 ± 0.0014	1.0196 ± 0.0008	(*27*)
Pentachloroethane	Cr(II)	-14.7 ± 0.6‰	1.0303 ± 0.0006	1.0149 ± 0.0006[a]	(*28*)
Hexachloroethane	Cr(II)	-10.4 ± 0.5‰	1.0212 ± 0.0005	1.0105 ± 0.0005[a]	(*28*)
γ-HCH	*D. gigas*	-4.0 ± 0.2‰	1.0246 ± 0.0012[a]	1.0121 ± 0.0006[a]	(*26*)
	D. multivorans	-3.4 ± 0.2‰	1.0208 ± 0.0012[a]	1.0103 ± 0.0006[a]	(*26*)

[a] AKIE values were approximated as described (21) from reported ε_{bulk} values.

7.5 References

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CHAPTER 8

U(VI) REDUCTION TO MONONUCLEAR U(IV) BY *DESULFITOBACTERIUM* SPP.

Reproduced in part with permission from Fletcher, K. E.; Boyanov, M. I.; Thomas, S. H.; Wu, Q.; Kemner, K. M.; Löffler, F. E. U(VI) reduction to mononuclear U(IV) by *Desulfitobacterium* spp. *Environ. Sci. Technol.* **2010**, *12*, 4705-4709.

8.1 Abstract

The bioreduction of U(VI) to U(IV) affects uranium mobility and fate in contaminated subsurface environments and is best understood in gram-negative model organisms such as *Geobacter* and *Shewanella* spp. In this study, we demonstrate that U(VI) reduction is a common trait of gram-positive *Desulfitobacterium* spp. Five different *Desulfitobacterium* isolates reduced 100 μM U(VI) to U(IV) in less than 10 days while U(VI) remained soluble in abiotic and heat-killed controls. U(VI) reduction in live cultures was confirmed using X-ray absorption near-edge structure (XANES) analysis. Interestingly, while bioreduction of U(VI) is almost always reported to yield the uraninite mineral (UO_2), extended X-ray absorption fine structure (EXAFS) analysis demonstrated that the U(IV) produced by the *Desulfitobacterium* spp. was not UO_2. The EXAFS data indicated that the U(IV) product was a phase or mineral composed of mononuclear U(IV) atoms closely surrounded by light element shells. This atomic arrangement likely results from inner-sphere bonds between U(IV) and C/N/O or P/S-containing ligands, such as carbonate or phosphate. The formation of a distinct U(IV) phase warrants further study

because the characteristics of the reduced material affect uranium stability and fate in the contaminated subsurface.

8.2 Introduction

Uranium processing has resulted in widespread environmental contamination, particularly at U.S. Department of Energy (DOE) sites. Oxidized hexavalent uranium, U(VI), is generally soluble and mobile, but forms sparingly soluble uraninite (UO_2) upon reduction to tetravalent uranium, U(IV) (1). Therefore, bioreduction of U(VI) to U(IV) is a promising approach to immobilize uranium in subsurface environments and prevent plume migration (2). Research on U(VI) reduction has focused on gram-negative model organisms that reduce U(VI) to UO_2 even though the ability to reduce U(VI) is distributed among numerous bacterial phyla, and recent observations suggest that not all bacteria produce UO_2 (1, 3-5). The mobility and long-term stability of uranium is influenced by the form of the reduced product; therefore, identifying and characterizing microbial U(VI) reduction products is vital for predicting U(IV) behavior *in situ* (3, 6-11).

At the uranium-contaminated DOE Integrated Field-Scale Subsurface Research Challenge (IFC) site in Oak Ridge, TN, organisms closely related to characterized metal reducers affiliated with both the gram-negative delta-Proteobacteria and the gram-positive Clostridia classes have been detected (12-14). Within the class Clostridia, *Clostridium*, *Desulfitobacterium*, and *Desulfosporosinus* populations are present at the Oak Ridge IFC site (12-14), and members of the *Clostridium* and *Desulfosporosinus*

groups have been shown to reduce U(VI) to U(IV) (15-16). *Desulfitobacterium* spp. are not recognized as U(VI) reducers and only one strain has been implicated in U(VI) reduction (17). *Desulfitobacterium* spp. are members of soil and subsurface microbial communities, but the commonality of U(VI) reduction among members of the *Desulfitobacterium* genus is unknown. The goal of this study was to determine if U(VI) reduction is a shared trait of *Desulfitobacterium* spp. and to characterize the reduced product.

8.3 Experimental Section

8.3.1 Bacterial Strains and Experimental Conditions

The isolates used in this study were *Desulfitobacterium chlororespirans* strain Co23 (18), *D. dehalogenans* strain JW/IU-DC1 (19), *D. hafniense* strain JH1 (20), *D.* sp. strain PCE1 (21), and *D.* sp. strain Viet1 (22). These strains were isolated in different laboratories from soil, sludge, and freshwater sediments obtained from geographically distinct locations. Defined, anaerobic, 30 mM bicarbonate-buffered mineral salts medium was prepared with a N_2/CO_2 (80%/20%, vol/vol) headspace as described (23), except that the phosphate concentration was reduced from 1.5 mM to 0.3 mM and sodium sulfide was omitted. Medium was amended with a vitamin solution (24) and 5 or 10 mM pyruvate. Pyruvate supported fermentative growth and served as the carbon source. Glass serum bottles with 60 or 160 mL nominal capacity contained approximately 30 or 100 mL of medium, respectively, and received 3% (vol/vol) inocula. When cultures became visibly turbid, the vessels were amended with 100 μM soluble U(VI) from a 30

mM uranyl carbonate stock prepared as described (23) and also received 2 mL H_2 (strains JH1 and Viet1) or 10 mM pyruvate (strains Co23, JW/IU-DC1, and PCE1) as electron donor. Following U(VI) amendment, cultures were incubated at room temperature in the dark without shaking. Strain Viet1 cultures grown with pyruvate and then incubated at 80°C for 15 minutes (which resulted in complete loss of viability based on previous experiments) served as killed controls. After cooling to room temperature in an ice bath, the cultures were amended with 100 μM U(VI) and electron donor. Uninoculated (i.e., abiotic) controls consisted of mineral salts medium amended with vitamins, 10 mM pyruvate, and 100 μM uranyl carbonate.

8.3.2 Uranium Quantification

All manipulations were performed using anoxic techniques or inside an anoxic chamber (Coy Laboratory Products, Ann Arbor, MI). Aqueous samples (1 mL) were removed from the bottles 1 hour after U(VI) amendment and periodically thereafter. Immediately after sample removal, 0.5 mL from each 1-mL sample was filtered through a 0.2 μm membrane syringe filter (Pall Corporation, East Hills, New York) or was centrifuged inside an anoxic chamber for 15 minutes at 10,000 rpm. Concentrations of U(VI) measured in the filtrate and supernatant were similar and, in some cases, below the detection limit of 5 μM, suggesting that both procedures removed insoluble uranium. Hence, the U(VI) concentrations measured in these samples correspond to the concentration of soluble U(VI). To verify the formation of U(IV), the remaining 0.5 mL sample volumes were transferred to sterile, 2-mL plastic tubes under oxic conditions (i.e., ambient air) and placed on a shaker at 150 rpm for a minimum of 1 hour to oxidize U(IV)

to U(VI) (23, 25-26). After shaking, these samples were filtered through 0.2 μm membrane syringe filters. Exposure of the samples to air resulted in U(IV) oxidation, and subsequent U(VI) measurements yielded total uranium concentrations. Soluble U(VI) was quantified by laser excitation spectrofluorescence with a luminescence spectrometer as previously described (27). Briefly, 0.1 mL aliquots from samples were diluted with 0.9 mL filtered, deionized water and amended with 30 μL of a 40 mM sodium hypophosphite and 80 mM sodium pyrophosphate solution. Nominal U(IV) concentrations were calculated by subtracting the concentration of soluble U(VI) from the nominal concentration of total uranium.

8.3.3 Characterization of Uranium Precipitates using X-Ray Absorption Spectroscopy

Uranium L_{III}-edge X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) analyses were performed to determine the valence state and the average local environment of uranium in the hydrated solid phase. Measurements were carried out at the MRCAT/EnviroCAT sector 10-ID (28), Advanced Photon Source (APS), Argonne National Laboratory, Illinois. Samples for XAFS analysis were mounted by filtering the suspensions through 0.22 μm membranes in an anoxic glove box. The membrane and solids were sealed in Kapton™ film (Dupont, Circleville, OH). Samples prepared in this manner have shown no oxidation changes under ambient atmosphere for at least 8 hours (29). The sealed sample holders were exposed to air only for about 30 seconds while being transferred from an O_2-free transport container to the N_2-purged detector housing. Beamline parameters have been published previously (30-31). Briefly, the beamline undulator was tapered and the

incident energy was scanned by using the Si(111) reflection of the double-crystal monochromator in quick-scanning mode (approximately 3 minutes per scan for the extended region and 40 seconds per scan for the near-edge region). Sample heterogeneity and beam-induced chemical changes were closely monitored and were not observed. The linearity of the experimental setup resulted in less than 0.1% change in the EXAFS signal when the incident intensity was attenuated by 50% (32). Data reduction and analysis were performed using the programs Autobk and Feffit (33-34).

An alkaline (pH 10.7) solution of U:carbonate (1:30, mole:mole) was used as a standard for carbonate-complexed U(VI) (speciation ~100% $UO_2(CO_3)_3$). U(IV) standards included a crystalline UO_2 (Alfa Aesar, Ward Hill, MA) diluted 1:100 in SiO_2 (35) and previously characterized U(IV) nanoparticles, produced either biogenically by *Shewanella oneidensis* MR-1 (26) or abiotically by green rust (29).

8.4 Results

8.4.1 U(VI) Reduction is a Common Trait of *Desulfitobacterium* spp.

In live strain Viet1 cultures, the concentration of soluble U(VI) decreased with incubation time and was below the detection limit of 5 μM 9 days after U(VI) amendment (Table 8.1, Figure 8.1). The amount of insoluble U(IV) increased concomitantly with the decrease in soluble U(VI) (Figure 8.1), indicating that the cells had reduced U(VI) to an insoluble form of U(IV). Similar observations were made in cultures of *Desulfitobacterium* sp. strain Co23, strain JH1, strain JW/IU-DC1, and strain PCE1. Within 7 days of U(VI) amendment, 90% of the initial U(VI) was removed from solution

while the nominal concentration of total uranium remained approximately constant (Table 8.1), indicating that soluble U(VI) was reduced to an insoluble U(IV) precipitate. In abiotic medium and heat-inactivated strain Viet1 controls, soluble U(VI) decreased negligibly, thus demonstrating that U(VI) reduction is a biotic process (Table 8.1, Figure 8.1).

Table 8.1 U(VI) reduction by *Desulfitobacterium* isolates. Reported values represent averages from duplicate (strain JW/IU-DC1 and strain PCE1) or triplicate vessels (strain Co23, strain JH1, strain Viet1, and controls).

	Incubation Time (days)	Percent Uranium Detected [a]		
		U(VI)	Total U	U(IV)
Controls				
Medium only (abiotic)	15	109.1 ± 10.7	97.4 ± 3.8	3.9 ± 3.4
Strain Viet1 (heat-inactivated)	9	88.9 ± 10.6	84.1 ± 21.0	6.2 ± 14.9
Live Cultures				
Strain Co23	3	BDL[b]	103.5 ± 10.8	111.7 ± 16.4
Strain JH1	7	9.6 ± 8.6	127.1 ± 15.4	135.5 ± 26.1
Strain JW/IU-DC1	3	BDL [b]	90.5 ± 5.5	118.4 ± 6.6
Strain PCE1	6	5.3 ± 7.4	118.5 ± 29.0	121.2 ± 9.2
Strain Viet1	9	BDL[b]	104.6 ± 19.7	108.4 ± 9.5

[a] The percent uranium detected was determined by dividing the amount of uranium measured at the incubation time (second column) by the amount of total uranium measured initially.
[b] BDL indicates that concentrations were below the detection limit of 5 µM.

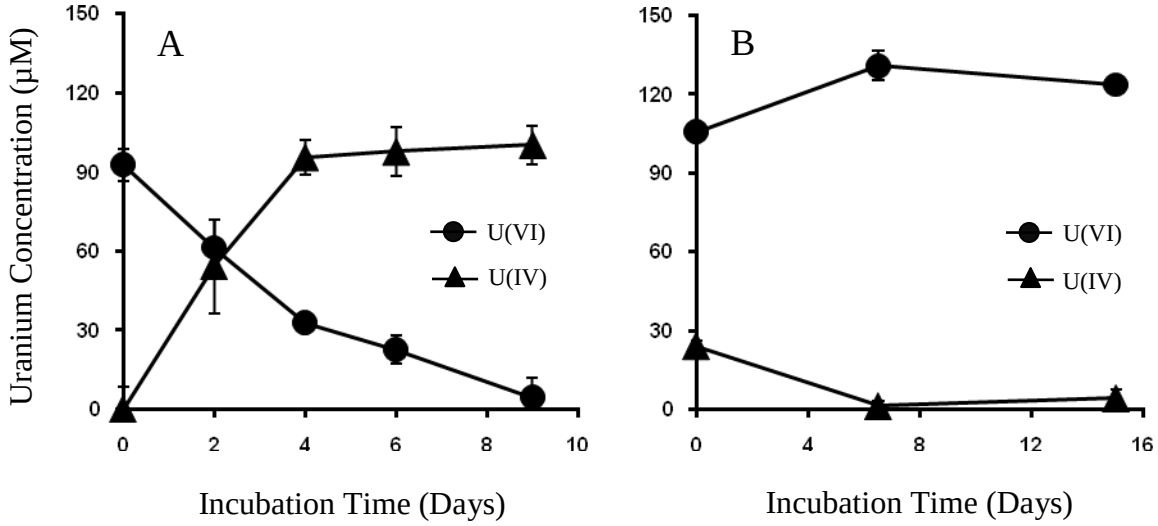

Figure 8.1 Soluble U(VI) and nominal U(IV) concentrations in live *Desulfitobacterium* sp. strain Viet1 cultures (A) and in abiotic controls (B). Data were averaged from triplicate cultures and error bars depict one standard deviation. In some cases, error bars are not visible because standard deviations are small.

8.4.2 XANES and EXAFS Analyses Confirm U(VI) Reduction and Demonstrate the Formation of Mononuclear U(IV)

XANES and EXAFS analyses performed on samples from all five live *Desulfitobacterium* cultures directly determined the average valence state and atomic environments of the solid phase uranium. The XANES spectra obtained from samples prepared from live *Desulfitobacterium* cultures demonstrated that at least 95% of all solid phase uranium was present as U(IV) (Figure 8.2), confirming the spectrofluorescence measurements and U(VI) reduction. Figure 8.3 shows that indistinguishable EXAFS spectra were obtained from all strains, indicating that all of the cultures generated the same U(IV) product. To further characterize the reduced product, the spectrum of the biogenic U(IV) produced in the *Desulfitobacterium* cultures was compared to U(IV) standards of known structure (Figure 8.3). The Fourier transformed (FT) EXAFS spectra of a crystalline UO_2 standard and a previously characterized nanoparticulate U(IV) phase (26) show a doublet between 3 and 4.2 Å (Figure 8.3). This spectral feature results from the edge-sharing U-U coordination in UO_2 at approximately 3.87 Å. The U(IV) produced in the *Desulfitobacterium* cultures does not show this doublet (Figure 8.3) and fits of the U(IV) spectra with the uraninite EXAFS model demonstrate the lack of a defined bidentate U(IV)-O_2-U(IV) coordination which is found in uraninite or coffinite [$U(SiO_4)_9(OH)_4$]. Consequently, the reduced U(IV) atoms are either individually bound to solid phase ligands or exist as molecular U(IV) minerals such as $CaU(PO_4)_2$ (36). The FT spectral feature observed between 2.4 and 3.6 Å in spectra obtained with the material

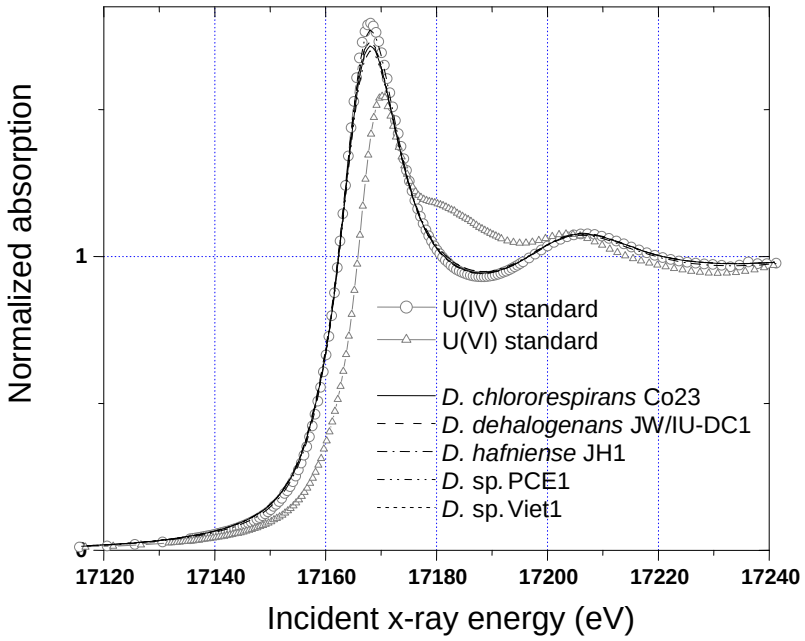

Figure 8.2 Uranium L_{III} edge XANES data obtained from the solid phase uranium in *Desulfitobacterium* cultures compared to completely reduced and oxidized standards. The data from the *Desulfitobacterium* cultures overlay each other and the U(IV) standard spectrum.

generated in the *Desulfitobacterium* cultures is not part of the main peak contribution at 1.8 Å from the near-neighbor O atoms. Modeling indicates consistency of the additional spectral feature with the presence of at least two light atom shells (such as C/N/O or S/P) at U(IV)-atom distances between 3.0 and 3.8 Å. Due to the small amplitude and overlapping peaks, the exact identity of the shells could not be unambiguously assigned. Nevertheless, the presence of the additional spectral structure, the ability to model the spectra with light atom shells, and the close distance of these shells suggest inner-sphere complexes of the U(IV) atoms with light element oxyanion ligands (e.g.,carbonate, carboxyl, phosphoryl).

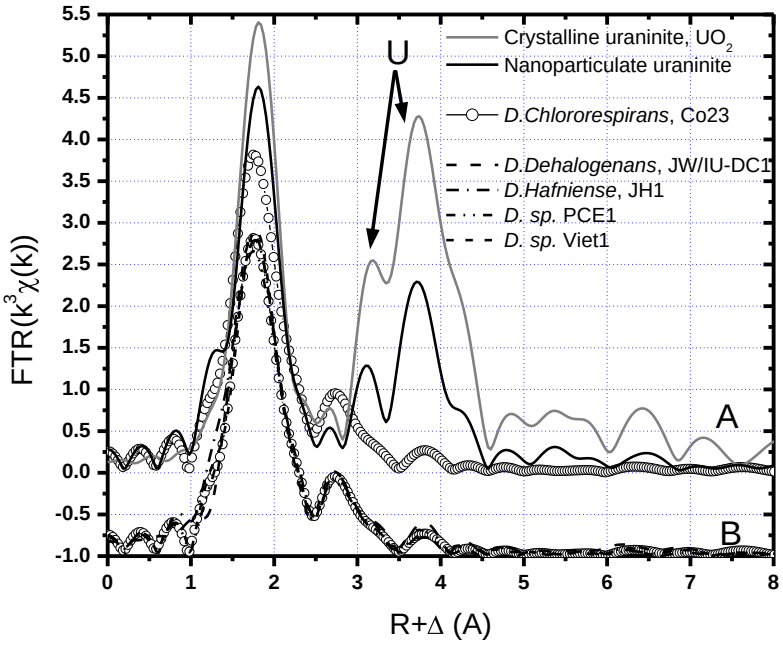

Figure 8.3 Uranium L_{III} edge EXAFS data of the U(IV) phase produced by strain Co23 (symbols) compared to crystalline UO_2 and nanoparticulate UO_2 and the U(IV) phases produced by four other *Desulfitobacterium* cultures. The spectral doublet resulting from U-U coordination in the UO_2 structure is indicated by the arrows. Data are k^3 weighed and FT over the range Δk=2.0-10.4 $Å^{-1}$ using a 1.0 Å Hanning window (34).

8.5 Discussion

Desulfitobacterium spp. are metabolically versatile anaerobic bacteria commonly present in soil, sediment, and subsurface environments and contribute to the reduction of oxidized metals and metalloids including arsenic, iron, manganese, and selenium (37-38). The ability of members of this bacterial genus to reduce radionuclides such as U(VI) has not been established. Our findings show that U(VI) reduction is a feature shared among *Desulfitobacterium* spp. and suggest that *Desulfitobacterium* spp. play important roles in controlling subsurface uranium mobility and fate. This is of particular importance as *Desulfitobacterium* have been detected at several uranium-impacted DOE sites (13-14, 39).

The product of microbial U(VI) reduction is almost always reported to be nanoparticulate UO_2 (7-11, 26, 40-41). Only in a few cases, U(VI) bioreduction has resulted in the formation of products other than UO_2. Specifically, Khijniak *et al*. (5) demonstrated that *Thermoterrabacterium ferriducens* produces ningyoite [$CaU(PO_4)_2$ x H_2O], Francis and Dodge (3) reported that *Clostridium* spp. produce a U(IV)-citrate complex, and Junier *et al*. (4) reported that UO_2 is likely not the dominant product of U(VI) reduction by spores of *Desulfotomaculum reducens* strain MI-1. Experiments assessing U(VI) microbial reduction typically use uranyl acetate or uranyl carbonate (7-9, 25-27, 41-43), but in the experiments performed with *T. ferriducens* and the *Clostridium* spp., U(VI) was provided as uramphite [$(NH_4)(UO_2)(PO_4)$ x $3H_2O$] and U(VI)-citrate, respectively (3, 5), which likely impacted the form of the produced U(IV). Although some *Desulfitobacterium* spp. were reported to produce spores, strains JH1, Viet1, and

PCE1 are not spore formers, indicating that vegetative cells were responsible for U(VI) reduction.

In contrast to most U(VI)-reducing organisms, including gram-negative model organisms such as *Anaeromyxobacter*, *Geobacter*, *Desulfovibrio*, and *Shewanella* (8, 11, 25, 42, 44), *Desulfitobacterium* spp. did not produce UO_2 but generated mononuclear U(IV). Biotic factors (e.g., electron transport machineries, cellular components, extracellular features) and abiotic factors (e.g., solution composition) can influence the nature of the reduced product. For example, a U(IV) phase different from UO_2 is produced by the chemical reduction of U(VI) by Fe(II) (30). Microbial U(VI) reduction yielding UO_2 has been observed in a variety of media with diverse solution compositions including bicarbonate-buffered groundwater (25) piperazine-N,N'-bis-(2-ethanesulfonic acid)-buffered artificial groundwater (26), 30 mM bicarbonate buffer (7-8, 26, 41, 43), and unbuffered water (16). The medium used in our *Desulfitobacterium* experiments was similar in composition to aqueous systems used in previous work that determined UO_2 as the reduced product, suggesting biological factors are involved; however, mononuclear U(IV) formation may be controlled by a complex interplay between biotic and abiotic (e.g., medium composition) factors, which future studies should explore in more detail.

Similarly to UO_2, the mononuclear U(IV) phase produced in *Desulfitobacterium* cultures is readily oxidized upon oxygen exposure (Table 8.1), but further characterization is needed to describe the stability and mobility of mononuclear U(IV) (e.g., the potential for complexation with organic ligands and colloidal transport). Comprehensive knowledge of the different processes and mechanisms involved in U(VI) reduction are crucial for making meaningful predictions about the mobility and fate of

uranium in the contaminated subsurface and for achieving lasting uranium immobilization *in situ*. Future studies assessing the biomolecular mechanisms of U(VI) reduction and identifying and characterizing the properties of the reduced product(s) are required to understand the contributions of *Desulfitobacterium* spp. to U(VI) immobilization.

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CHAPTER 9

CONCLUSIONS, ENGINEERING OUTCOMES, SCIENTIFC ADVANCES, AND RECOMMENDATIONS

9.1 Chapter Overview

Chapter 9 describes the key practical and scientific contributions to the fields of chlorinated compound and radionuclide bioremediation. Section 9.2 contains a brief review of the main conclusions and is followed by a discussion of the impact of the key findings on the practice of bioremediation. Section 9.4 addresses how the work has advanced scientific understanding. The final section of Chapter 9 provides suggestions for future research.

9.2 Key Findings

The overall goal of the research presented here was to increase understanding of the microbes that catalyze key reactions for the detoxification of chlorinated solvents and the immobilization of radionuclides. Populations catalyzing these reactions were identified and characterized based on their biochemistry, ecology, and physiology both in order to contribute to scientific understanding of microbiology and to optimize bioremediation strategies employed by environmental engineers.

The six key finding presented in this dissertation are:

1. Thermal treatment increases the quantity of available electron donor, but in some cases, biostimulation will still be required to fuel complete dechlorination of chlorinated ethenes to ethene due to total electron donor demand.
2. Contrary to a number of reports, *Clostridium bifermentans* strain DPH-1 is not capable of PCE dechlorination and it is, in fact, a *Desulfitobacterium hafniense* population that catalyzes the dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) in this co-culture.
3. Whereas identified *Dhc* populations are unlikely to catalyze complete reductive dechlorination of chlorinated ethenes to ethene at elevated temperatures, some *Dhc* remain viable during incubation at inhibitory temperatures and produce elevated levels of reductive dehalogenase (RDase) gene transcripts as a stress response.
4. Because isotope effects for the dechlorination of *cis*-DCE and VC are similar for all tested *Dhc* pure and mixed cultures, accurately estimating biodegradation extents based on compound specific stable isotope analysis (CSIA) may be feasible.
5. Dichloroelimination of 1,2-dichloropropane (1,2-D) by two unique *Dhc* populations produces statistically identical isotope effects that are similar to the effects measured for other dichloroelimination reactions catalyzed by *Dhc*.
6. Reduction of oxidized U(VI) is a widespread trait of *Desulfitobacterium* although the reduced U(IV) product is not the commonly reported uraninite (UO_2).

9.3 Impacts for Remediation Practice

The key findings of this dissertation apply to three topics within the practice of remediation: i) the coupling of thermal treatment with bioremediation for the clean-up of sites contaminated with chlorinated ethenes, ii) the in situ monitoring of dechlorination reactions catalyzed by *Dhc*, and iii) the immobilization of U(VI) via microbial reduction. Although identified *Dhc* are not capable of maintaining activity at elevated temperatures, interest has recently developed in coupling bioremediation and thermal treatment because thermal treatment rapidly removes significant chlorinated ethene mass and increases available electron donor. Results from laboratory experiments confirmed that currently available dechlorinating communities are not capable of activity at the elevated temperatures typically present in the source zone during thermal treatment; however, *Dhc* can maintain activity at moderately elevated temperatures of up to 40°C. Moderately elevated temperatures are present in the perimeter of the source zone during thermal treatment and therefore, dechlorination may continue in the perimeter of the source zone as long as electron donor (i.e., hydrogen) is available. Results from microcosm studies established that the electron donor released during thermal treatment is not always adequate to fuel complete reductive dechlorination of chlorinated ethenes to ethene, particularly if the bioaugmentation inoculum includes methanogens. Therefore, in some cases, biostimulation must be employed to fuel complete, rapid dechlorination even when bioremediation is coupled to thermal treatment.

Quantification of *Dhc* 16S rRNA and RDase genes is the most widely employed technique for the monitoring of *Dhc* in situ. Recently, interest has developed in employing quantification of *Dhc* gene transcripts as well. However, laboratory experiments demonstrated that whereas *Dhc* gene abundances correlated with dechlorination activity, *Dhc* gene transcription was up-regulated as a stress response to inhibitory conditions. Therefore, elevated levels of *Dhc* gene transcripts should not be interpreted as an indicator of metabolic activity in all cases. In fact, traditional measurements of gene abundances may actually provide a more accurate assessment of in situ dechlorination.

Another technique for monitoring in situ dechlorination is compounds specific isotope analysis (CSIA). CSIA can be employed both to qualitatively assess in situ dechlorination and to quantitatively estimate the biodegradation extent based on enrichment (ε) factors determined in laboratory experiments. At many sites, the dechlorination intermediates *cis*-DCE and VC accumulate, but a thorough study of the range of ε factors for dechlorination of these compounds had never been conducted. Results from laboratory studies showed that isotope effects for *cis*-DCE and VC dechlorination are relatively consistent across all *Dhc* strains regardless of the specific RDase catalyzing the reaction. Therefore, at sites with significant accumulation of dechlorination intermediates, estimates of the extent of dechlorination can be generated based on a small range of similar ε factors. Because ε factors for the dichloroelimination of 1,2-D had never been reported, it was unknown whether CSIA could be employed even to qualitatively assess in situ biodegradation of 1,2-D. Laboratory studies revealed that 1,2-D dechlorination by two distinct *Dhc* populations produced statistically identical

isotope effects, suggesting that both the qualitative and quantitative assessment of 1,2-D biodegradation in situ is feasible. Although CSIA may serve as a powerful tool for estimating the fraction of a compound that has undergone biodegradation in situ, to estimate the dechlorination extent at a field site, a considerable number of samples must collected and analyzed to ensure that representative isotope compositions are measured. Further, in order to accurately quantify isotope compositions, adequate compound concentrations must be present and the detection limit for isotope ratio mass spectrometers is typically lower than that of gas chromatographs equipped with FID detectors.

The most widely studied means for the immobilization of uranium is the reduction of U(VI) to U(IV) as UO_2 by gram negative Proteobacteria. Interestingly, results established that U(VI) reduction is widespread in the gram positive *Desulfitobacterium* genus and that the reduced U(IV) does not precipitate as UO_2. For the immobilization of uranium plumes, these results have significant practical implications. First, when evaluating methods to enhance bioreduction of U(VI), the response of *Desulfitobacterium* as well as gram negative U(VI)-reducing organisms must be considered. Second, the effects of ionic strength, pH, and redox conditions on all U(IV) precipitates, rather than only UO_2, must be considered when predicting in situ uranium stability and mobility.

9.4 Advancement to Scientific Understanding

The key findings of this dissertation have advanced scientific understanding by i) correcting the scientific literature, ii) contributing to the understanding of *Dhc* biology,

and iii) increasing knowledge of U(VI) reduction. Over five peer-reviewed publications regarding dechlorination of PCE by a pure culture of *Clostridium bifermentans* strain DPH-1 have been published. These publications have been cited more than 30 times, but, when carefully evaluated, some of the results presented suggested that the strain DPH-1 culture was not pure. By demonstrating and reporting in a peer-reviewed journal that the *Clostridium* culture was actually a mixed culture containing both *Clostridium* and *Desulfitobacterium* populations, my research corrected an error that had been propagated throughout the scientific literature on reductive dechlorination of chlorinated ethenes.

Dhc are mainly studied because they are the key organisms for the reductive dechlorination of chlorinated ethenes, but *Dhc* are also of interest because they are deeply-branching and have highly restricted metabolisms as well as small genomes. Laboratory experiments demonstrated increased expression of some *Dhc* genes involved in organohalide respiration during incubation at inhibitory, elevated temperatures, suggesting that up-regulation is a stress response to heat. Understanding the response of *Dhc* cells to stress is vital for determining their basic survival mechanisms and their regulatory network. The metabolism of *Dhc* is solely reliant on dehalogenation, but the biochemical transformation mechanisms associated with these reactions are not completely understood. *Dhc* RDases were examined based on their isotope effects. Although some *Dhc* strains catalyze the same reaction with unique RDases, the isotope effects for individual dechlorination reactions catalyzed by unique *Dhc* populations are highly similar. In fact, the isotope effects for two unique dichloroelimination reactions (i.e., dichloroelimination of 1,2-D and dichloroelimination of 1,2-dichloroethane (1,2-

DCA)) were also similar. These results suggest that differences in *Dhc* RDases may not fundamentally affect dechlorination mechanisms.

U(VI) reduction is an important process not only for the immobilization of uranium plumes in groundwater, but also because U(VI) can serve as a terminal electron acceptor in bacterial metabolism. Uranium chemistry is complex, but only 4 forms of reduced uranium have been described and the majority of studies have focused on the production of UO_2 by gram negative Proteobacteria. The demonstration that U(VI) reduction is a shared trait of *Desulfitobacterium* isolates and that the product of U(VI) reduction by *Desulfitobacterium* is not UO_2 expanded the scientific understanding of both the bacteria responsible for U(VI) reduction and the possible products of U(VI) reduction.

9.5 Recommendations for Future Research

Multiple laboratory studies have investigated the potential benefits and drawbacks of coupling thermal treatment with bioremediation for the remediation of chlorinated ethene contaminated sites, but most studies have relied on batch systems. At contaminated sites, groundwater flow affects the transport of chlorinated solvents, electron donor, and dechlorinators. Therefore, future experiments should evaluate thermal treatment coupled to bioremediation in flow-through column systems.

Monitoring in situ dechlorination is complex, but vital for demonstrating biodegradation. The identification of RDases catalyzing dechlorination reactions and the development of molecular techniques to quantify RDase genes or gene transcripts has

proven to be a powerful tool for monitoring *Dhc*; however, current techniques cannot be reliably used to differentiate actively dechlorinating, viable cells from dead or inhibited cells. Therefore, future research should focus on the identification of *Dhc* genes that are only transcribed during active dechlorination activity. Whereas results from laboratory experiments strongly suggests that CSIA can be used to quantitatively evaluate the extent of biodegradation of *cis*-DCE, VC, and 1,2-D, the accuracy of CSIA needs to be verified in situ.

Biodegradation of chlorinated solvents results in the destruction of the toxic compound. Conversely, as uranium is a non-degradable element, it can only be immobilized by bioremediation. Currently, it is unclear whether the production of mononuclear U(IV) rather than UO_2 was due to abiotic or biotic factors, but both the mononuclear U(IV) phase produced in *Desulfitobacterium* cultures and UO_2 are readily oxidized upon exposure to oxygen. Because the form of the reduced product determines not only the potential for uranium re-solubilization, but also the potential for uranium complexation and transport, future studies should evaluate the role that solution composition (e.g., the growth medium) versus biology played in the production of this unique U(IV) product. In order to control uranium plumes, studies should focus on manipulating either U(VI) reducers or subsurface conditions to facilitate the production of stable U(IV) products.

APPENDIX A

EXPERIMENTAL PROCEDURES

A.1 Anaerobic Minimal Medium

1. Prepare the 100x DCB-1 salt solution by dissolving 100 g NaCl, 50 g $MgCl_2$ x $6H_2O$, 20 g KH_2PO_4, 30 g NH_4Cl, 30 g KCl, and 1.5 g $CaCl_2$ x $2H_2O$ in approximately 700 mL of e-pure water. Dissolve each chemical completely prior to addition of the next chemical. Once all chemicals have been dissolved, add e-pure water until the final volume is 1 L.
2. Prepare the DCB-1 trace element solution by dissolving 1.5 g $FeCl_2$ x $4H_2O$, 0.19 g $CoCl_2$ x 6 H_2O, 0.1 g $MnCl_2$ x 4 H_2O, 70 mg $ZnCl_2$, 6 mg H_3BO_3, 36 mg Na_2MoO_4 x 2 H_2O, 24 mg $NiCl_2$ x 6 H_2O, and 2 mg $CuCl_2$ x 2 H_2O in approximately 700 mL of e-pure water and 10 mL of 25% (wt/wt) HCl. Dissolve each chemical completely prior to addition of the next chemical. Once all chemicals have been dissolved, add e-pure water until the final volume is 1 L.
3. Prepare the DCB-1 Se/Wo solution by dissolving 6 mg Na_2SeO_3 x 5 H_2O, 8 mg Na_2WO_4 x 2 H_2O, and 0.5 g NaOH in approximately 700 mL of e-pure water. Dissolve each chemical completely prior to the addition of the next chemical. Once all chemicals have been dissolved, add e-pure water until the final volume is 1 L.
4. Prepare the DCB-1 resazurin solution by dissolving 1 g resazurin sodium salt (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) in approximately 700 mL of e-

pure water. Once the resazurin has completely dissolved, add e-pure water until the final volume is 1 L.

5. Measure out 990 mL of deionized water.
6. Add approximately half of the water to a large round bottom flask and begin to stir using a magnetic stir bar.
7. Add 10 mL of 100x DCB-1 salt solution, 1 mL of the trace element solution, 1 mL of the Se/Wo solution, and 0.25 mL of the resazurin solution to the flask.
8. Add the rest of the water and mix the medium for at least 30 seconds.
9. Under a stream of N_2 gas, bring the medium to a boil for a minimum of 10 minutes. Attach a condenser to the flask to avoid loss of volume.
10. Under a stream of N_2 gas, cool the medium to room temperature by placing the flask in an ice bucket.
11. Once the medium is at room temperature, under a stream of N_2 gas, remove the flask from the ice bucket and place it on a magnetic stir plate.
12. Change the stream of N_2 gas to a stream of O_2-free 80% N_2 / 20% CO_2 (vol/vol) gas. Bubble the gas through the medium.
13. Add 0.035 g L-cysteine (0.2 mM) and 2.50 g $NaHCO_3$ (30 mM) to the medium and stir using a magnetic stir bar.
14. While continuing to bubble the gas mixture through the medium, measure the pH of the medium using a glass electrode. Adjust the pH to 7.2 - 7.3 by increasing or decreasing the relative volume of CO_2 gas (increase CO_2 volume to decrease solution pH).

15. Once the pH has been adjusted, stop bubbling the gas mixture through the medium, but continue to flush the flask headspace with the gas mixuture. Remove the pH electrode.
16. Add 0.048 g Na_2S x 9 H_2O to the medium and stir for at least 2 minutes.
17. Stop stirring the medium and begin flushing three 160 mL (nominal volume) serum bottles with the gas mixture (continue flushing the medium as well).
18. Add 100 mL of medium from the flask to a flushed serum bottle.
19. Carefully remove the gas line from the serum bottle while simultaneously sealing the bottle using a stopper.
20. Move the gas line to a new serum bottle and continue dispensing medium to serum bottles.
21. Crimp serum bottles
22. Incubate serum bottles overnight at room temperature.
23. Autoclave all clear serum bottles and discard bottles that are pink or blue.

Reference:

(1) Fletcher, K. E.; Ritalahti, K. M.; Pennell, K. D.; Takamizawa, K.; Löffler, F. E. Resolution of culture *Clostridium bifermentans* DPH-1 into two populations: A *Clostridium* sp. and tetrachloroethene (PCE) dechlorinating *Desulfitobacterium hafniense* strain JH1. *Appl*. *Environ*. *Microbiol*. **2008**, *74*, 6141-6143.

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A.2 Compound Specific Isotope Analysis

1. Add 1 mL of 1 M NaOH to a 10 mL serum bottle.
2. Collect a 7 mL aqueous sample of culture suspension.
3. Add the 7 mL aqueous sample to the 10 mL serum bottle and, immediately after sample addition, seal the bottle with a Teflon-lined, butyl rubber stopper.
4. Crimp the bottle and shake vigorously for 2 seconds.
5. Store the bottle at 4°C prior to analysis.
6. Immediately prior to analysis, warm the sample bottle to at least room temperature (24°C).
7. Remove a gaseous headspace sample (0.05 to 1.00 mL) and immediately inject the sample into a gas chromatograph (GC) combustion isotope ratio mass spectrometer.
8. Analyze at least two gaseous headspace samples from each sample bottle.

References:

(1) Fletcher, K. E.; Löffler, F. E.; Richnow, H. H.; Nijenhuis, I. Stable carbon isotope fractionation of 1,2-dichloropropane during dichloroelimination by *Dehalococcoides* populations. *Environ. Sci. Technol.* **2009**, *43*, (18), 6915-6919.

(2) Lee, P. K. H.; Conrad, M. E.; Alvarez-Cohen, L. Stable carbon isotope fractionation of chloroethenes by dehalorespiring isolates. *Environ. Sci. Technol.* **2007**, *41*, (12), 4277-4285.

A.3 Dechlorination Activity Assays

1. Prepare the following anoxic stocks: 1 M potassium acetate (pH 5.8), 95 mM titanium citrate (see section A.9), 100 mM methyl viologen (Sigma Aldrich, St. Louis, MO), 100 mg/L of the chlorinated compound of interest dissolved in e-pure water, and e-pure water.
2. Autoclave all solutions.
3. Transfer all solutions and a 5 mL serum bottle with Teflon-lined, butyl rubber stopper into the glove box.
4. In the glove box, combine solutions in the 5 mL vial to final concentrations of 100 mM potassium acetate, 4 mM titatanium citrate, 4 mM methyl viologen, and 25 mg/L chlorinated compound (Table A.3.1).
5. Add proteins, cell extracts, or whole cells (Table A.3.1).

Reference:

(1) Hölscher, T.; Görisch, H.; Adrian, L. Reductive dehalogenation of chlorobenzene congeners in cell extracts of *Dehalococcoides* sp. strain CBDB1. *Appl. Environ. Microbiol.* **2003**, *69*, (5), 2999-3001.

Table A.3.1 Concentrations of dechlorination activity assay stock solutions and volumes of stocks required for an assay with a total volume of 4 mL.

Component	Stock Concentration	Volume Added to Assay (µL)
Potassium Acetate (pH 5.8)	1 M	400
Titanium Citrate	95 mM	170
Methyl Viologen	100 mM	160
Diluted Chlorinated Compound	100 mg/L	1000
E-pure Water	NA	1270
Dhc Pure Culture	10^6 or more cells/mL	1000

A.4 Gas Chromatography

1. Seal a clean, dry 20 mL vial with a Teflon-lined, butyl rubber stopper.
2. Collect a 1 mL aqueous sample and immediately inject the sample into the 20 mL vial.
3. Add 1 mL of deionized water to two 20 mL vials and seal the vials with Teflon-lined, butyl rubber stoppers.
4. Load one deionized water sample into the Hewlett-Packard (HP) 7694 headspace autosampler at position 1.
5. Load the sample of interest in the autosampler at position 2.
6. Load the remaining deionized water sample into the autosampler at position 3.
7. Ensure that the autosampler conditions are as follows:
    a. Carrier gas pressure of 2 psig,
    b. Cycle time of 14 minutes,
    c. Injection time of 0.5 minutes,
    d. Loop equilibration time of 0.05 minutes,
    e. Loop fill time of 0.03 minutes,
    f. Oven temperature of 70°C,
    g. Pressurization time of 0.5 minutes,
    h. Sample loop temperature of 125°C,
    i. Vial equilibration time of 15 minutes using maximum agitation, and
    j. Vial pressurization of 10 psig.

8. Ensure that the autosampler is connected to the HP 6890 GC equipped with a HP-624 column (60 m by 0.32 mm and 1.8 µm nominal film thickness) and a flame ionization detector.
9. Ensure that the GC conditions are as follows:
    a. Column flow at 3.0 mL/min,
    b. Column pressure at 23.11 psig,
    c. FID temperature of 280°C,
    d. FID gases are air (400 mL/min), helium (27 mL/min), and hydrogen (30 mL/min),
    e. Inlet flow of 5.7 mL/min,
    f. Inlet pressure of 23.11 psig,
    g. Inlet split ratio of 0.1:1,
    h. Inlet temperature of 200°C, and
    i. Oven temperature of 60°C for 2 minutes followed by an increase to 200°C at 25°C/min.
10. Press the start buttons on the GC and autosampler.

References:

(1) Amos, B. K.; Christ, J. A.; Abriola, L. M.; Pennell, K. D.; Löffler, F. E. Experimental evaluation and mathematical modeling of microbially enhanced tetrachloroethene (PCE) dissolution. *Environ. Sci. Technol.* **2007**, *41*, (3), 963-970.

(2) Fletcher, K. E.; Löffler, F. E.; Richnow, H. H.; Nijenhuis, I. Stable carbon isotope fractionation of 1,2-dichloropropane during dichloroelimination by *Dehalococcoides* populations. *Environ. Sci. Technol.* **2009**, *43*, (18), 6915-6919.

A.5 Gas Chromatography Standards of Gaseous Compounds

1. Prepare aerobic DCB-1 medium by combining appropriate volumes of salt, trace element, Se/Wo, and resazurin solutions with deionized water, reductants, and buffers.
2. Dispense 100 mL of aerobic DCB-1 medium into 6 dry 160 mL serum bottles.
3. Seal serum bottles with Teflon-lined, butyl rubber stopper.
4. Via syringe, add 0, 1, 2, 3, 4, or 6 mL of the gaseous compound of interest to each of the 160 mL serum bottles.
5. Add air to each serum bottle such that the sum of the added gaseous volumes is 6 mL.
6. Place the serum bottles on a shaker overnight.
7. Measure compound concentrations using gas chromatography (see section A.4).
8. Determine how peak area corresponds to known compound concentration.

A.6 Gas Chromatography Standards of Liquid Compounds

1. Prepare the stock solution as follows:
    a. Weigh a dry, stoppered 10 mL vial,
    b. Add a total of 10 mL of methanol and the compound of interest to the 10 mL vial and weigh the vial following the addition of each component, and
    c. Vortex the stoppered vial for at least 2 minutes.
2. Prepare aerobic DCB-1 medium by combining appropriate volumes of salt, trace element, Se/Wo, and resazurin solutions with deionized water, reductants, and buffers.
3. Weigh 6 dry 160 mL serum bottles sealed with Teflon-lined, gray butyl rubber septa.
4. Dispense 100 mL of aerobic DCB-1 medium into each bottle and weigh bottles again.
5. In a cold room (4°C), dispense 1.0, 0.8, 0.6, 0.4, 0.2, or 0.0 mL of the stock solution to each of the 160 mL serum bottles.
6. Weigh the serum bottles.
7. Add methanol to each serum bottle such that the sum of the added stock solution and the methanol is 1.0 mL.
8. Weigh the serum bottles.
9. Place the serum bottles on a shaker overnight.
10. Measure compound concentrations using gas chromatography (see section A.4).
11. Determine how peak area corresponds to known compound concentration (Figure A.6.1).

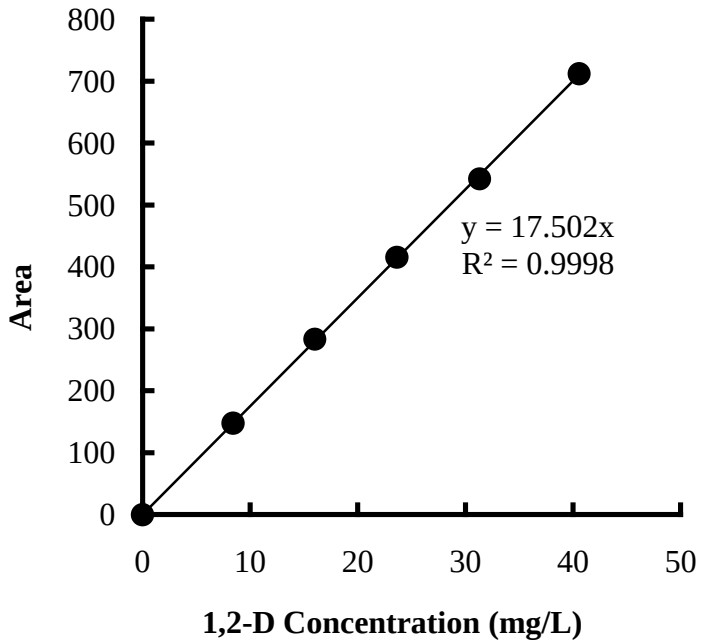

Figure A.6.1 The relationship between known 1,2-D concentrations (mg/L) and peak areas measured using gas chromatography.

A.7 High Performance Liquid Chromatography (HPLC)

1. Remove a 1 mL aqueous sample and immediately filter the sample through a 13 mm syringe filter with 0.2 µM nylon membrane.
2. Combine 475 µL filtered sample with 25 µL 1 M H_2SO_4 in a 1 mL glass HPLC vial and close the HPLC vial with a white plastic cap.
3. Combine 475 µL e-pure water with 25 µL 1 M H_2SO_4 in a 1 mL glass HPLC vial and close the HPLC vial with a white plastic cap.
4. Load the e-pure water sample to position 1 in the Waters 717 plus autosampler and load the sample of interest to position 2.
5. Ensure that the Waters autosampler is connected to the Waters HPLC system and that the Waters 2487 dual-wavelength absorbance detector is set to 210 nm.
6. Press the run button on the Waters HPLC system.

References:

(1) Fletcher, K. E.; Ritalahti, K. M.; Pennell, K. D.; Takamizawa, K.; Löffler, F. E. Resolution of culture *Clostridium bifermentans* DPH-1 into two populations: A *Clostridium* sp. and tetrachloroethene (PCE) dechlorinating *Desulfitobacterium hafniense* strain JH1. *Appl*. *Environ*. *Microbiol*. **2008**, *74*, 6141-6143.

(2) Sung, Y.; Fletcher, K. E.; Ritalahti, K. M.; Apkarian, R. P.; Ramos-Hernández, N.; Sanford, R. A.; Mesbah, N. M.; Löffler, F. E. *Geobacter lovleyi* sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium. *Appl. Environ. Microbiol.* **2006**, *72*, (4), 2775-2782.

A.8 Pyrolusite Coated Sand

1. Dispense 2.6 kg of Federal Fine sand into a dry 4L Pyrex baking dish.
2. Add 1 L of 1N nitric acid solution to the sand and mix the sand and acid solution with a glass rod.
3. Cover the sand and acid with a Nalgene lid and incubate for 2 days at room temperature.
4. Rinse the sand by dispensing 500 g aliquots of sand into a 140 mesh stainless steel sieve and flushing with deionized water for 5 minutes.
5. Transfer 1.5 kg of wet, rinsed sand into a fresh 4 L Pyrex baking dish.
6. Place the sand in an oven at 105°C until the sand appears dry.
7. Once the sand has been cooled to 24°C, add 100 mL of 45 - 50% (wt%) $Mn(NO_3)_2$ solution.
8. Mix the sand and manganese with a glass rod for 5 minutes until the sand appears evenly coated.
9. Place the sand in the oven at 105°C and stir periodically with the glass rod.
10. Bake the sand overnight.
11. Break up clumps of sand with the glass rod and increase the oven temperature to 160°C. Bake overnight.
12. Cool the sand to 24°C and transfer 1.3 kg of sand to a 1L Erlenmyer flask.
13. Rinse the sand with deionized water until dark particles are no longer present in the rinse water.

References:

(1) Jardine, P.M.; Taylor, D.L. Kinetics and mechanisms of Co(II)EDTA oxidation by pyrolusite. *Cosmochim*. *Acta*. **1995**, *59*, 4193-4203.

(2) Stahl, R.S.; James, B.R. Zinc sorption by manganese-oxide-coated sand as a function of pH. *Soil Sci*. *Am*. *J*. **1991**, *55*, 1291-1294.

A.9 Titanium Citrate

1. Prepare a saturated $NaHCO_3$ solution as follows:
   a. Add 30 mL deionized water and 3 - 4 g of $NaHCO_3$ to a 50 mL pyrex bottle.
   b. Stir the solution using a magnetic stir bar.
   c. After 10 minutes of stirring, remove the solution from the stir plate and allow the undissolved $NaHCO_3$ to settle to the bottom of the bottle.
2. Combine 2.94 g sodium citrate (molecular weight (MW) of 294.1 g/mole) with deionized water to a final volume of 50 mL.
3. Pour the 0.2 M sodium citrate solution into a 160 mL serum bottle.
4. Bubble N_2 gas through the sodium citrate solution while stirring using a magnetic stir bar.
5. After 15 minutes of bubbling with N_2 gas, seal the sodium citrate solution with a butyl rubber stopper.
6. Transfer the sodium citrate solution into the glove box.
7. In the glove box, add 5 mL of aqueous 15% $TiCl_3$ dissolved in 10% hydrochloric acid (Sigma Aldrich, St. Louis, MO) to the sodium citrate solution.
8. Stopper the serum bottle and transfer the titanium citrate solution out of the glove box.
9. Remove the stopper from the serum bottle and quickly begin sparging the titanium citrate solution with CO_2 gas while stirring.
10. Add 7 mL of the saturated $NaHCO_3$ solution to the titanium citrate solution.

11. Measure the pH of the titanium citrate solution using indicator paper. Add saturated $NaHCO_3$ solution until the pH is approximately 7 (approximately 7 mL are required).

12. Stopper the titanium citrate and autoclave.

References:

(1) Fletcher, K. E.; Löffler, F. E.; Richnow, H. H.; Nijenhuis, I. Stable carbon isotope fractionation of 1,2-dichloropropane during dichloroelimination by *Dehalococcoides* populations. *Environ. Sci. Technol.* **2009**, *43*, (18), 6915-6919.

(2) Zehnder, A. J. B.; Wuhrmann, K. Titanium (III) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. *Science* **1976**, *194*, 1165-1166.

A.10 Uranium Quantification

1. Remove a 1 mL aqueous sample and immediately filter 0.5 mL through a 13 mm syringe filter with a 0.2 µM nylon membrane. This is referred to as the "reduced sample."
2. Place the other 0.5 mL in a plastic 2-mL Eppendorf tube. This is referred to as the "oxidized sample."
3. Store the reduced sample at -20°C until immediately prior to analysis.
4. Shake the oxidized sample overnight.
5. After shaking, filter the oxidized sample through a 13 mm syringe filter with a 0.2 µM nylon membrane.
6. Store the oxidized sample at -20°C until immediately prior to analysis.
7. Immediately prior to analysis, thaw both reduced and oxidized samples.
8. In an Eppendorf tube, combine 900 µL e-pure water, 100 µL sample, and 30 µL of a 40 mM sodium hypophosphite and 80 mM sodium pyrophosphate solution.
9. Vortex Eppendorf tubes for 5 seconds.
10. Quantify U(VI) in samples using laser excitation spectrofluorescence with a luminescence spectrometer at a wavelength of 498.5 nm.

References:

(1) Fletcher, K. E.; Boyanov, M. I.; Thomas, S. H.; Wu, Q.; Kemner, K. M.; Löffler, F.E. U(VI) reduction to mononuclear U(IV) by *Desulfitobacterium* spp. *Environ. Sci. Technol.* **2010**, *12*, 4705-4709.

(2) Wu, Q.; Sanford, R. A.; Löffler, F. E. Uranium(VI) reduction by *Anaeromyxobacter dehalogenans* strain 2CP-C. *Appl. Environ. Microbiol.* **2006**, *72*, (5), 3608-3614.

A.11 RNA Extraction and Reverse Transcription

1. At 4°C, collect biomass into a cell pellet. (From mixed cultures containing *Dehalococcoides* (*Dhc*), collect 5 to 10 mL of culture).
2. At 4°C, suspend the cell pellet in 500 µL of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA).
3. Vortex the suspended cells and incubate at room temperature for 5 minutes.
4. Centrifuge the suspended cells at 10,000 x g for 10 minutes at room temperature.
5. Remove the supernatant and store the cell pellet at -80°C if DNA and RNA cannot be extracted immediately.
6. Immediately prior to DNA and RNA extraction, thaw the cell pellet on ice.
7. Suspend the cells in 250 µL Tris-EDTA (pH 8) with 15 mg/mL lysozyme (Sigma Aldrich).
8. Add 1 µL 10% (wt/vol) SDS (Sigma Aldrich) to the suspended cells.
9. Add 600 µL of RLT Buffer (from the Allprep DNA/RNA Mini Kit (Qiagen)) with 6 µL β-mercaptoethanol (Sigma Aldrich) to the cell suspension.
10. Vortex the cell suspension for 5 minutes.
11. Extract DNA and RNA using the Allprep DNA/RNA Mini Kit following the manufacturer's instructions with the following modification
    a. Store the AllPrep DNA spin column at 4°C while RNA is being extracted.
    b. Once RNA has been extracted, immediately store samples at -80°C.
12. Remove the RNA samples from -80°C and place on ice to thaw.
13. Once RNA samples have thawed, add 20 U DNase I from the TURBO DNA-Free Kit (Ambion, Austin TX) per mL of RNA.

14. Add enough 10x DNase Buffer from the TURBO DNA-Free Kit for the final solution concentration to be 1x.
15. Incubate the samples at 37°C for 30 minutes.
16. Add 1 volume phenol solution (Sigma Aldrich item no. P4557) to the RNA sample and manually shake the sample for 1 minute.
17. Centrifuge the RNA sample for 5 minutes at 9,300 x g at room temperature.
18. Transfer the upper aqueous phase to a new tube.
19. Add 1 volume 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma Aldrich item no. 77617) and manually shake for 1 minute.
20. Centrifuge the RNA sample for 5 minutes at 9,300 x g at room temperature.
21. Transfer the upper aqueous phase to a new tube.
22. Add 0.1 volumes of 3M sodium acetate and mix by vortexing.
23. Add 2.5 volumes of ice cold 100% ethanol and mix by vortexing.
24. Store samples at -20°C for a minimum of 4 hours and a maximum of overnight.
25. Centrifuge samples at 12,000 x g for 25 minutes at 4°C.
26. Invert open microcentrifuge tubes and discard supernatant.
27. Add 750 µL ice cold 70% ethanol.
28. Invert tubes repeatedly to mix.
29. Centrifuge at 12,000 x g for 25 minutes at 4°C.
30. Invert open microcentrifuge tubes and discard supernatant.
31. Air dry RNA sample for 10-20 minutes at room temperature.
32. Dissolve the RNA sample into 20 µL diethylprocarbonate (DEPC) treated water (Invitrogen, Carlsbad, CA) with 1 µL RNase inhibitor (Qiagen).

33. Store RNA at -80°C.
34. Ensure that RNA is not contaminated by DNA by using the RNA sample as the template in a PCR reaction.
35. Evaporate samples in a SpeedVac for 30 minutes to reduce sample volume to less than 5 µL.
36. Add 1 µL of Hex primers, 1 µL of dNTPs, and 3 µL of DEPC treated water to the RNA sample (Invitrogen). Mix by pipetting gently.
37. Add DEPC treated water to obtain a final total volume of 10 µL.
38. Place samples at 65°C for 5 minutes.
39. Place samples on ice.
40. From the Superscript First Strand Synthesis System for RT-PCR (Invitrogen) add 4 µL $MgCl_2$, 2 µL 10x Buffer, 2 µL dithiothreitol (DTT), and 1 µL RNase inhibitor. Mix by pipetting gently.
41. Incubate samples at 25°C for 2 minutes.
42. Add 1 µL of Superscript II RT from the Superscript First Strand Synthesis System for RT-PCR and mix by pipetting gently.
43. Incubate samples at 25°C for 10 minutes.
44. Incubate samples on ice for 2 minutes.
45. Incubate samples at 42°C for 50 minutes.
46. Incubate samples at 70°C for 15 minutes.
47. Incubate samples on ice for 5 minutes.
48. Add 1 µL RNase H from the Superscript First Strand Synthesis System for RT-PCR.
49. Incubate samples at 37°C for 20 minutes.

50. RNA samples are now cDNA samples. Store samples at -20°C.

References:

(1) Amos, B. K.; Ritalahti, K. M.; Cruz-Garcia, C.; Padilla-Crespo, E.; Löffler, F. E. Oxygen effect on *Dehalococcoides* viability and biomarker quantification. *Environ. Sci. Technol.* **2008**, *42*, (15), 5718-5726.

(2) Sambrook, J.; Russell, D. W. *Molecular cloning: a laboratory manual*; Third ed.; Cole Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001; Vol. 3.

A.12 Denaturing Gel Electrophoresis (SDS-PAGE)

1. Cast or purchase a sodium dodecyl sulfate (SDS) 10% resolving and 4% stacking gel. Gels can be cast following the instructions provided in the Mini-PROTEAN Tetra Cell manual (Bio-Rad Hercules, CA).
2. Prepare the SDS sample buffer by combining 1.25 mL 0.5 M Tris-HCl (pH 8), 2.5 mL glycerol, 2.0 mL 10% (wt/vol) SDS, 0.2 mL 0.5% (wt/vol) bromophenol blue, 50 mM DTT, and deionized water to a total volume of 9.5 mL.
3. Prepare 10x SDS running buffer by combining 30.3 g Tris base, 144.0 g glycine, 10.0 g SDS and deionized water to a final volume of 1 L.
4. Prepare 2x SDS running buffer by diluting 10x SDS running buffer in deionized water.
5. Combine 10 µL protein samples with 10 µL 2x SDS running buffer and vortex to mix.
6. Load the samples to the gel and run the gel with SDS running buffer at 100 V for approximately 90 minutes.
7. Stain the gel using the silver stain method described by Nesterenko et al., 1994.

Reference:

(1) Adrian, L.; Rahnenführer, J.; Gobom, J.; Hölscher, T. Identfication of a chlorobenzene reductive dehalogenase in *Dehalococcoides* sp. strain CBDB1. *Appl. Environ. Microbiol.* **2007**, *73*, 7717-7724.

(2) Nesterenko, M. V.; Tilley, M.; Upton, S. J. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. *J. Biochem. Biophys. Methods* **1994**, *28*, 239-242.

A.13 Native Gel Electrophoresis

1. Collect biomass and store the cell pellet at -80°C. (From pure or highly enriched *Dhc* cultures, collect 500 mL of culture under anoxic conditions).
2. Using the 4x Native PAGE Sample Buffer and digitonin solutions included in the NativePAGE™ Sample Prep Kit (Invitrogen), prepare a 1x buffer and 1% digitonin solution.
3. Sparge the buffer and digitonin solution with N_2 gas briefly.
4. In a glove box, resuspend the *Dhc* pellet in 250 µL of the buffer and digitonin solution.
5. Add approximately 0.1 g of sterile, acid-washed 75 µm diameter glass beads (Sigma Aldrich) to the sample and bead beat for 10 seconds. Place the sample on ice immediately after bead beating.
6. Centrifuge the sample at 12,000 x g for 10 minutes at 4°C.
7. Discard the pellet and add 9.5 µL of G-250 (included in the NativePAGE™ Sample Prep Kit) to the supernatant.
8. Store the sample on ice while preparing the gel and running buffers.
9. In a cold room (approximately 10°C), rinse a NativePAGE™ Novex® 4-16% Bis-Tris gel (Invitrogen) with 4°C 1x Anode Buffer prepared from the NativePAGE™ Running Buffer Kit (Invitrogen).
10. In a cold room, place the gel in the XCell SureLock® Mini Cell (Invitrogen) and add 4°C 1x Anode Buffer to the cell.
11. In a cold room room, load 20 µL of sample per lane to the gel. Each sample must be loaded to 3 lanes so that proteins from the first lane can be stained, proteins from the

second lane can be used in activity assays, and proteins from the third lane can be separated further using SDS-PAGE.

12. In a cold room, add 1x Dark Blue Cathode Buffer prepared from the NativePAGE™ Running Buffer Kit to the cell.
13. In a cold room, run the gel at 150 V for 60 minutes, 250 V for 30 minutes, and 300 V for 10 minutes.
14. Separate individual lanes using a scalpal and stain only one lane per sample using the silver stain method described by Nesterenko et al., 1994. While one lane is being stained, leave the other lanes in the cold room soaking in the Anode Buffer.
15. To use separated proteins in activity assays:
    a. Use a scalpal to excise regions of the gel of interest and slice the excised gel into cubes that are as small as possible,
    b. Place gel cubes into activity assay vials (see section A.3),
    c. Transfer vials into a glove box and add activity assay solutions to vials, and
    d. Incubate activity assays anaerobically for at least 24 hours prior to GC analysis.
16. To separate proteins further using SDS PAGE,:
    a. Use a scalpel to excise regions of the gel of interst and slice the excised gel into cubes that are as small as possible,
    b. Add gel cubes to a microcentrifuge tube containing 250 µL SDS Elution Buffer (pH 7.0, 100 mM Tris-HCl and 0.1% (wt/vol) SDS),
    c. Shake microcentrifuge tubes overnight at room temperature,
    d. Centrifuge samples at 12,000 x g for 5 seconds, and

e. Load the sample supernatant into a Millipore Centrifugal Amicon Ultra Filter Unit (0.5 mL, 10K) and follow the manufacturer's instructions to concentrate the sample.

References:

(1) Adrian, L.; Rahnenführer, J.; Gobom, J.; Hölscher, T. Identification of a chlorobenzene reductive dehalogenase in *Dehalococcoides* sp. strain CBDB1. *Appl*. *Environ*. *Microbiol*. **2007**, *73*, 7717-7724.

(2) Nesterenko, M. V.; Tilley, M.; Upton, S. J. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. *J*. *Biochem*. *Biophys*. *Methods* **1994**, *28*, 239-242.

APPENDIX B

ADDITIONAL EXPERIMENTAL DATA

B.1 Dechlorination in Ft. Lewis and Great Lakes Microcosms

The results of experiments conducted with microcosms constructed from the Ft. Lewis and Great Lakes sites are described in Chapter 3. Due to the large quantity of data, plots showing the stepwise conversion of tetrachloroethene (PCE) or trichloroethene (TCE) to ethene were not included in this chapter. The stoichiometric conversion of TCE to ethene in Ft. Lewis microcosms incubated at 35°C prior to cooling to 24°C and bioaugmentation is shown in Figure B.1.1. Similarly, the conversion of PCE to ethene in bioaugmented Great Lakes microcosms consistently incubated at 24°C is shown in Figure B.1.2. In both figures, the effect of biostimulation is clearly evident based on the enhancement of dechlorination activity that occurs following biostimulation. The stepwise dechlorination shown in Figures B.1.1 and B.1.2 is representative of the general trends observed in the Ft. Lewis and Great Lakes microcosms.

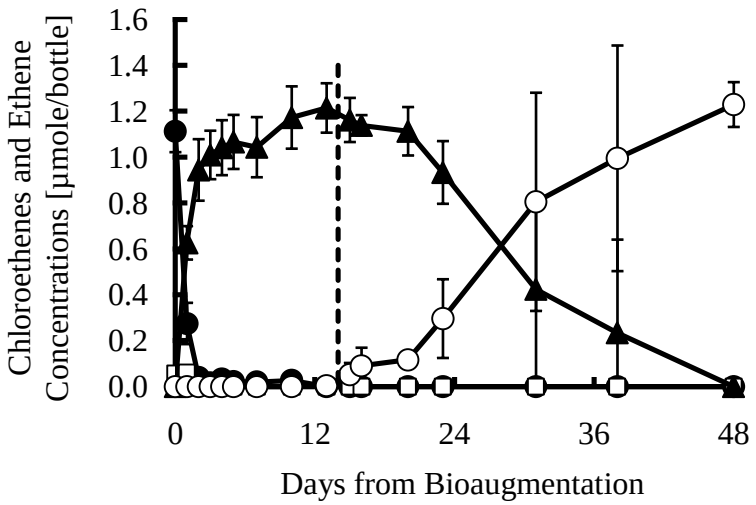

Figure B.1.1 Dechlorination of TCE in Ft. Lewis microcosms incubated at 35°C prior to cooling to 24°C and bioaugmentation. TCE (solid circles) dechlorination to *cis*-DCE (open squares), VC (solid triangles), and ethene (open circles). The vertical dashed line indicates the biostimulation event and error bars depict one standard deviation.

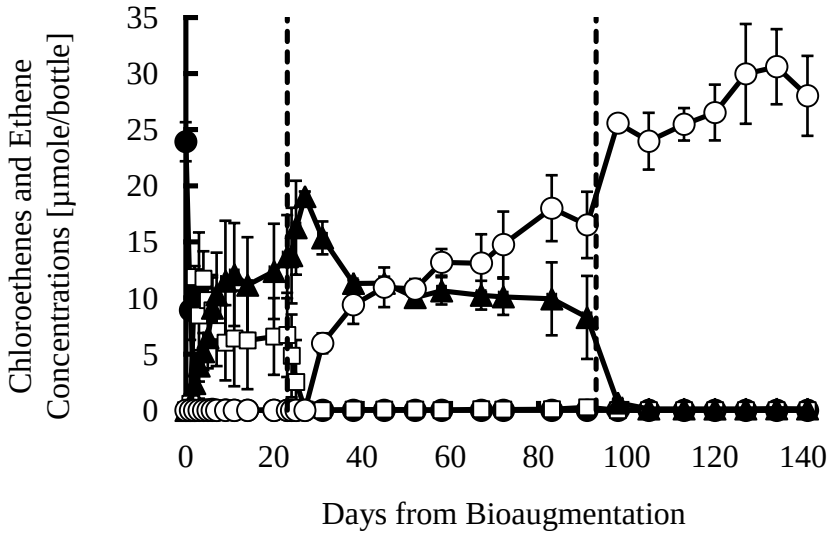

Figure B.1.2 Dechlorination of PCE in Great Lakes microcosms bioaugmented and consistently incubated at 24°C. PCE (solid circles) dechlorination to *cis*-DCE (open squares), VC (solid triangles), and ethene (open circles). TCE is not shown as TCE concentrations were below 1 µmole/bottle after 2 days of incubation. Vertical dashed lines indicate biostimulation events and error bars depict one standard deviation.

B.2 Chlorinated Ethene Fractionation

The results of experiments conducted with *Dehalococcoides* (*Dhc*) pure and mixed cultures to determine ε_{bulk} factors for dechlorinaiton of chlorinated ethenes are described in Chapter 6. Due to the large volume of data, Rayleigh plots are not presented in Chapter 6. Figures B.2.1 through B.2.5 show Rayleigh plots for the dechlorination of TCE, 1,1-dichloroethene (1,1-DCE), *cis*-1,2-dichloroethene (*cis*-DCE), *trans*-1,2-dichloroethene (*trans*-DCE), and vinyl chloride (VC). Details regarding culture and medium preparation, analytical techniques, sample collection, and isotope fractionation calcuations are presetented in Chapter 6.

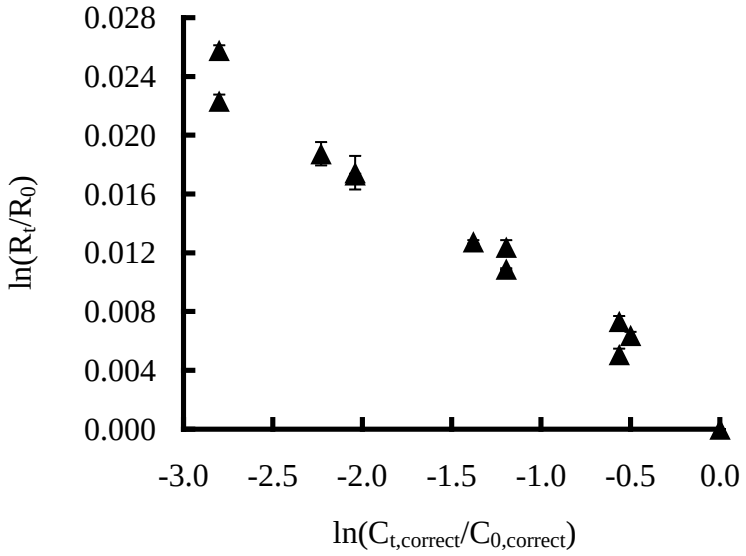

Figure B.2.1 Rayleigh plot showing TCE transformation by *Dhc* strain FL2. Error bars depict one standard deviation for duplicate or triplicate isotope measurements.

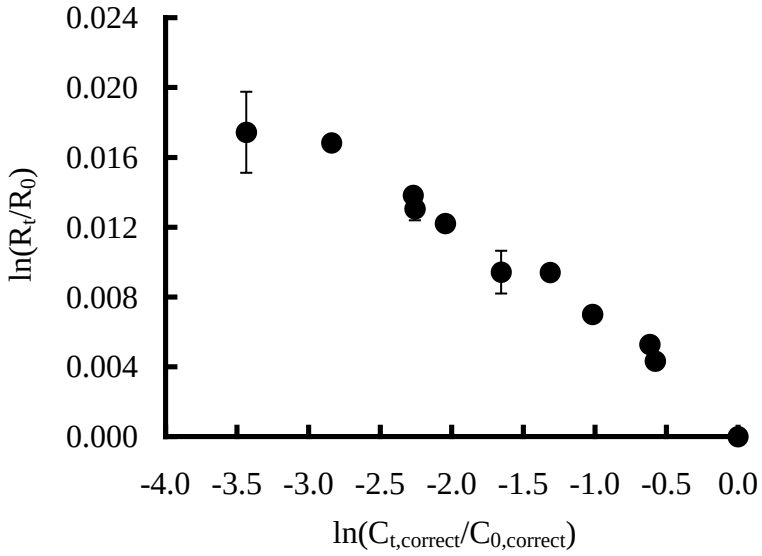

Figure B.2.2 Rayleigh plot showing 1,1-DCE transformation by *Dhc* strain BAV1. Error bars depict one standard deviation for duplicate or triplicate isotope measurements. In some cases, error bars are not visible because standard deviations are small.

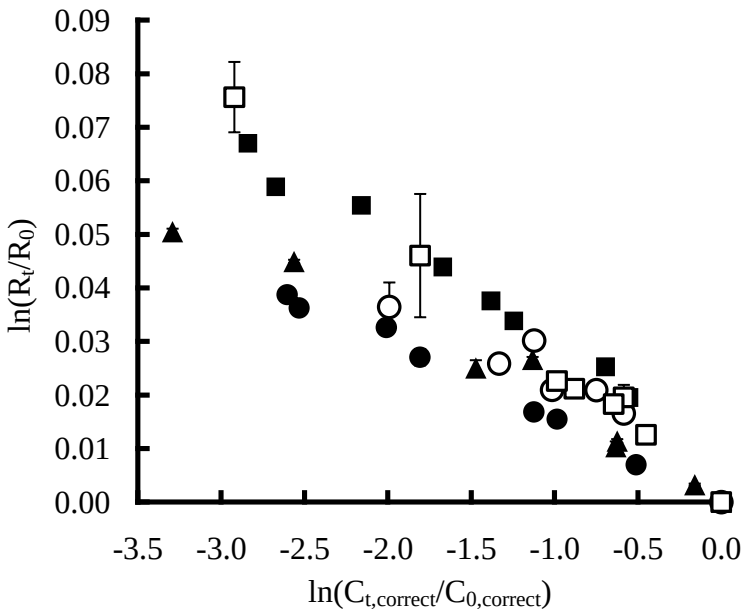

Figure B.2.3 Rayleigh plot showing *cis*-DCE transformation by *Dhc* strain BAV1 (filled circles), strain FL2 (filled triangles), strain GT (filled squares), strain VS (open circles), and consortium BDI (open squares). Error bars depict one standard deviation for duplicate or triplicate isotope measurements. In some cases, error bars are not visible because standard deviations are small.

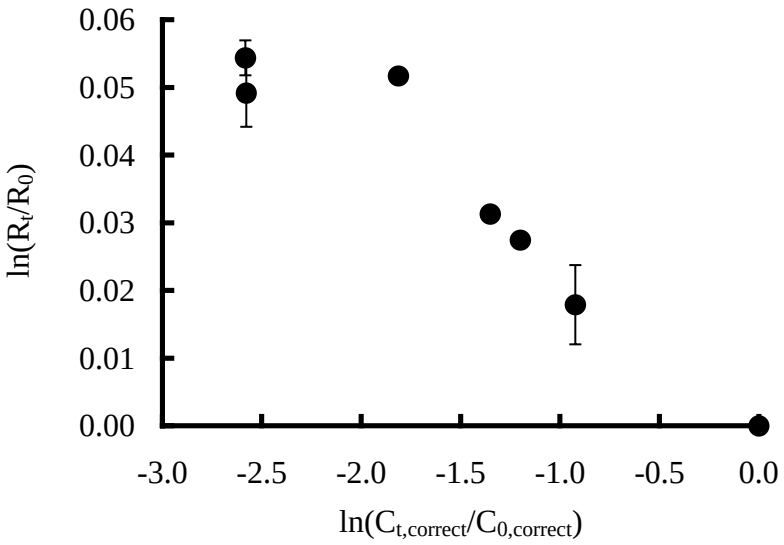

Figure B.2.4 Rayleigh plot showing *trans*-DCE transformation by *Dhc* strain BAV1. Error bars depict one standard deviation for duplicate or triplicate isotope measurements. In some cases, error bars are not visible because standard deviations are small.

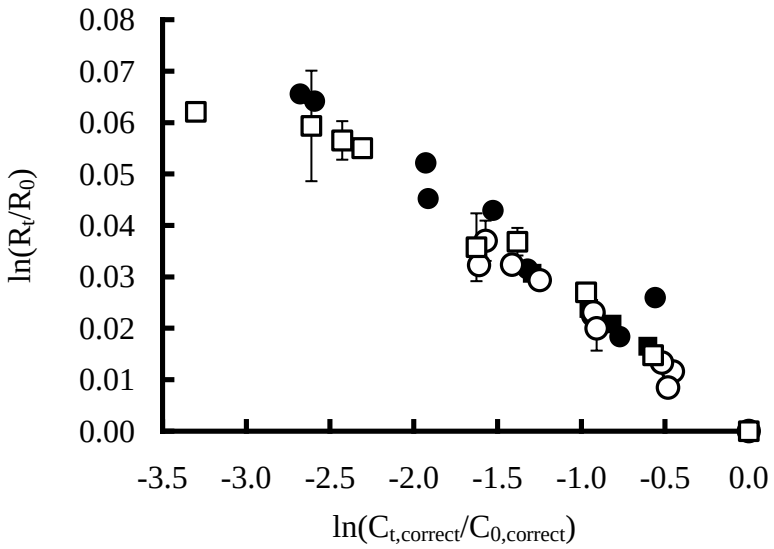

Figure B.2.5 Rayleigh plot showing VC transformation by *Dhc* strain BAV1 (filled circles), strain GT (filled squares), strain VS (open circles), and consortium BDI (open squares). Error bars depict one standard deviation for duplicate or triplicate isotope measurements. In some cases, error bars are not visible because standard deviations are small.

B.3 The Effect of Phosphate on U(VI) Solubility and Quantification

The results of experiments assessing the U(VI) reduction capabilities of *Desulfitobacterium* isolates were described in Chapter 8. As described in section 8.3.2, in order to quantify both U(VI) and total uranium, aqueous samples removed from cultures were divided into two subsamples. One subsample was filtered immediately after collection and uranium concentrations measured in these subsamples correspond to the concentration of soluble U(VI). The other subsample was oxidized prior to filtration. When U(IV) is oxidized, it reverts to soluble U(VI) and therefore, the uranium concentrations measured in these subsamples correspond to the nominal total uranium concentration (assuming that no U(VI) has precipitated). Because U(VI) may precipitate abiotically, an experiment was conducted to ensure that the sampling technique could be used to distinguish U(VI) precipitation from U(VI) reduction.

Uranium (100 μM) was amended to serum bottles containing uninoculated medium (as described in section 8.3.1) and phosphate-amended medium. Phosphate-amended medium contained 10 mM K_2HPO_4, which is known to promote abiotic U(VI) precipitation as U(VI)-phosphate. In uninoculated medium, neither the concentration of soluble U(VI) nor the nominal total uranium concentration decreased during 15 hours of incubation (Figure B.3.1). In phosphate-amended medium, both concentrations of soluble U(VI) and nominal concentrations of total uranium decreased to below the detection limit within 10 hours of incubation (Figure B.3.1). The decrease in the nominal concentration of total uranium indicates that the disappearance of soluble U(VI) was not due to U(VI) reduction, but rather due to U(VI) precipitation, presumably as U(VI)-

phosphate. These results demonstrate that with the analysis of both anoxic and oxidized subsamples, U(VI) precipitates can be differentiated from reduced U(IV) precipitates.

B.4 Soluble U(VI) in Heat-Inactivated *Desulfitobacterium* Cultures

In Chapter 8, the results of experiments assessing the U(VI) reduction capabilities of *Desulfitobacterium* isolates were described. As a control, soluble U(VI) and total nominal uranium concentrations were also measured in cultures containing heat-inactivated *Desulfitobacterium* sp. strain Viet1 cells; however, due to the large volume of data presented in Chapter 8, figures demonstrating the persistance of soluble U(VI) in heat-inactivated control cultures were not presented. Strain Viet1 cells were inactivated via incubation at 80°C for 15 minutes as described in section 8.3.1. After 9 days of incubation in heat-inactivated cultures, soluble U(VI) persisted at concentrations similar to those of nominal total uranium, yielding negligable nominal concentrations of reduced U(IV) (Figure B.4.1). Conversely, after 9 days of incubation in live strain Viet1 cultures, soluble U(VI) concentrations decreased to below the detection limit (5 μM) while the nominal concentration of total urnanium remained constant, indicating that reduced U(IV) was produced (Figure 8.1).

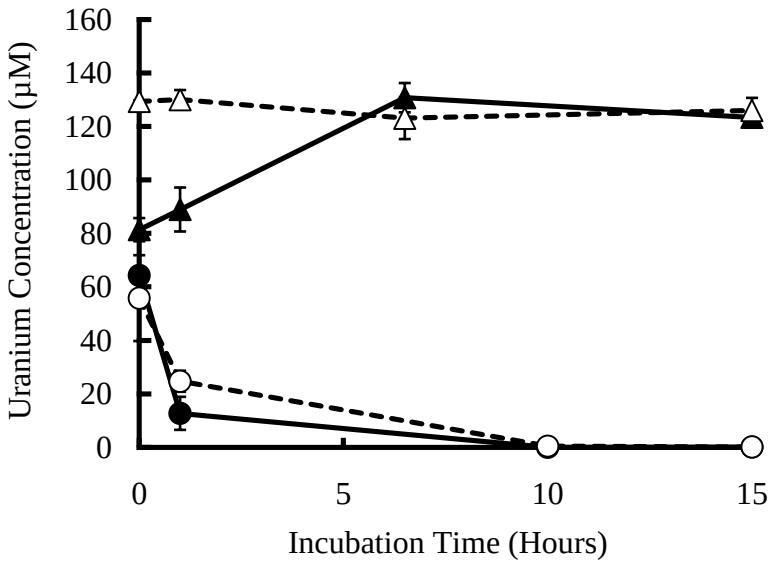

Figure B.3.1 Soluble U(VI) (filled symbols) and nominal total uranium (open symbols) concentrations in uninoculated medium (triangles) and in phosphate-amended medium (circles). Data were averaged from triplicate cultures and error bars depict one standard deviation.

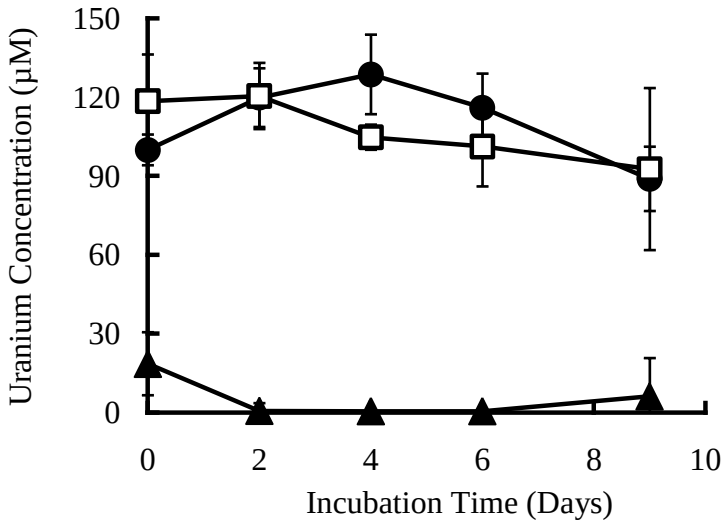

Figure B.4.1 Soluble U(VI) (filled circles), nominal total uranium (open squares), and reduced U(IV) concentrations (filled triangles) in heat-inactivated strain Viet1 cultures. Data were averaged from triplicate cultures and error bars depict one standard deviation.